IMPROVING THE NUTRITIVE VALUE OF FLOUR

II. FURTHER STUDIES ON THE EFFECT OF SUPPLEMENTING ENRICHED FLOUR WITH B-COMPLEX VITAMINS AND SOME OBSERVATIONS ON THE USE OF 80% EXTRACTION FLOUR[1]

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FOUR FIGURES AND TWO PLATES

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Previous work by Westerman and Hall ('47) has shown that when enriched patent flour was used as the sole source of the B-complex vitamins in the diet of the rat, supplements of certain B vitamins improved the nutritive value of the flour. This has been partially confirmed, particularly in regard to riboflavin, by Guerrant and Fardig ('47).

In the experiments reported by Westerman and Hall ('47) the vitamins were added to the flour in amounts similar to those found in whole wheat in order to replace the vitamins lost in the milling process. However, none of the animals made as good growth as those on the stock diet. This was probably due to an inadequate supply of certain vitamins. In a normal diet the amount of B vitamins in whole wheat, if fed at a 30% or 40% level in the diet, would not be expected to meet the body's needs for these vitamins. However, among the low income groups of the population the diets may be inadequate in meat, milk and vegetables. These materials furnish the best sources of the B vitamins and in many cases are partially replaced by the use of larger amounts of cereals in the

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diets. Therefore the question arises as to whether the addition of more of the B vitamins or increasing the amounts of those vitamins already added in the enriching process would prove beneficial. It was decided to conduct a series of experiments, based primarily on the rat's requirements for the B-complex vitamins, using whole wheat and enriched flour supplemented with different amounts of the B vitamins, to determine the effect on growth, maintenance, reproduction and length of life.

In 1946 the government decided that 80% extraction flour would be used for a period of time in this country. This was done in order to have more of the wheat berry included in the flour and to make a larger supply of flour available for human food. Some observations on the use of 80% extraction flour as a source of the B vitamins in the diet are included in this report.

EXPERIMENTAL PROCEDURE

The procedure described by Westerman and Hall ('47) was followed. Young albino rats weighing between 40 and 50 gm, of known hereditary and nutritional background, were placed on a B-complex free basal diet for 12 days to deplete their bodies of these vitamins. At the end of the depletion period the animals were divided into groups in such a manner as to have equal distribution in regard to sex and litter mates. Forty per cent of the sucrose in the basal diet was replaced by flour or ground whole wheat unless otherwise indicated. Four different experiments were conducted. The composition of all the diets is shown in table 1.

RESULTS AND DISCUSSION

First experiment: Enriched flour included in the diets at a 40% level with B vitamins added

In the first experiment diet I had choline and pyridoxine added to the enriched flour and diet II had choline, pyridoxine and riboflavin added (table 1). The analysis of the enriched flour showed that it contained 7.82 μg per gram of thiamine and 2.69 μg per gram of riboflavin. It supplied 18.77 μg of

TABLE 1

Composition of the diets

The B-complex free basal diet consisted of 20% vitamin free casein, 60% sucrose, 12% fat, 5% salt mixture and 3% cod liver oil. The flour replaced an equivalent amount of sucrose and was fed at a 40% level in the diet except where indicated. The B vitamins were added to the diet as indicated below.

DIET NO.	CHANGES IN DIET			
	Experiment I	Experiment II	Experiment III	Experiment IV
I	Enriched flour + 0.75 mg choline + 2 μg pyridoxine per gram	Whole wheat	Whole wheat	Whole wheat
II	Enriched flour + 0.75 mg choline + 2 μg pyridoxine + 14.2 μg riboflavin per gram	Enriched flour	Whole wheat + 4.4 μg thiamine per gram	80% extraction flour
III		Enriched flour + 0.75 mg choline + 2 μg pyridoxine per gram	Enriched flour	80% extraction flour, enriched
IV		Enriched flour + 1 mg liver extract per gram	60% whole wheat	80% extraction flour, blended
V		Enriched flour + 0.75 mg choline + 2 μg pyridoxine + 14.2 μg riboflavin per gram	60% enriched flour	80% extraction flour, blended and enriched
VI		Enriched flour + 0.75 mg choline + 2 μg pyridoxine + 20.5 μg Ca pantothenate per gram	Enriched flour + 0.75 mg choline + 2 μg pyridoxine + 20.5 μg Ca pantothenate + 14.2 μg riboflavin per gram	Enriched flour
VII		Stock diet	Enriched flour + 0.75 mg choline + 2 μg pyridoxine + 20.5 μg Ca pantothenate + 14.2 μg riboflavin + 4.4 μg thiamine per gram	

thiamine and 6.46 μg of riboflavin per rat per day. Sure ('38) found that 10 μg of thiamine per day will meet the growth requirements of the rat. According to Edgar, Macrae and Vivanco ('37) the rat needs 40 μg per day of riboflavin for growth. Since the 6.46 μg of riboflavin furnished by the flour in diet II was not enough to support growth, it was decided to add 14.2 μg of riboflavin per gram to the enriched flour, thereby bringing the riboflavin intake per day up to 40.54 μg.

According to Tepley, Strong and Elvehjem ('42) flour contains approximately 3.5 μg per gram of pyridoxine. The flour in the diet provided a daily intake of 8.4 μg of pyridoxine. Lepkovsky ('38) reported that 10 μg of pyridoxine daily is necessary for optimal growth, so 2 μg of pyridoxine per gram was added to the flour. This would provide a total of 13.2 μg of pyridoxine per day, which should be ample for growth purposes.

The 0.75 mg of choline per gram was added to the flour to take care of any lack of this material in the diet. Griffith ('41) reported that with 18–24% of casein in the diet, hemorrhagic degeneration could be prevented by including 1–2 mg of choline in the daily ration. The diets used in these experiments allowed an intake of 1.7 mg choline per day. Dann ('41) reported that the rat synthesizes enough nicotinic acid for its needs, and Hundley ('47) found that tryptophane appeared to be a precursor of nicotinic acid. With casein in the diet to furnish tryptophane and the enriched flour providing nicotinic acid it was decided not to add this vitamin to the diet.

After the test was started the animals were weighed every 6 days over a 96-day period. The results are shown by the growth curves in figure 1. It will be noted that the animals on diet II, with the added riboflavin, made greater weight gains than those on diet I. The fur and general appearance of these animals were much better than those on diet I. While the addition of choline and pyridoxine to the flour may have had some effect in increasing the growth rate, the addition of a larger amount of riboflavin to the enriched flour along with

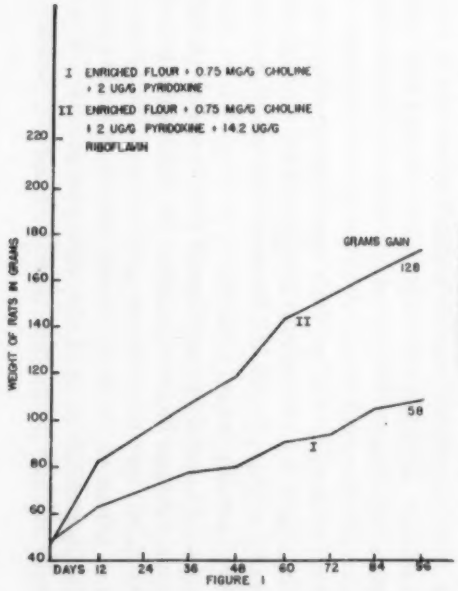
I ENRICHED FLOUR + 0.75 MG/G CHOLINE + 2 UG/G PYRIDOXINE
II ENRICHED FLOUR + 0.75 MG/G CHOLINE + 2 UG/G PYRIDOXINE + 14.2 UG/G RIBOFLAVIN
GRAMS GAIN
128
58
II
I
WEIGHT OF RATS IN GRAMS
DAYS 12 24 36 48 60 72 84 96

FIGURE 1

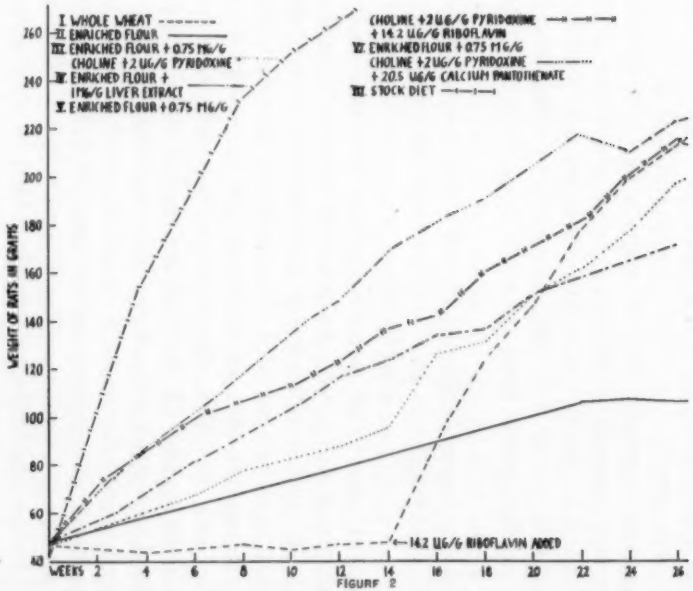
I WHOLE WHEAT
II ENRICHED FLOUR
III ENRICHED FLOUR + 0.75 MG/G CHOLINE + 2 UG/G PYRIDOXINE
IV ENRICHED FLOUR + 1 MG/G LIVER EXTRACT
V ENRICHED FLOUR + 0.75 MG/G CHOLINE + 2 UG/G PYRIDOXINE + 14.2 UG/G RIBOFLAVIN
VI ENRICHED FLOUR + 0.75 MG/G CHOLINE + 2 UG/G PYRIDOXINE + 20.5 UG/G CALCIUM PANTOTHENATE
VII STOCK DIET
← 14.2 UG/G RIBOFLAVIN ADDED
WEIGHT OF RATS IN GRAMS
WEEKS 2 4 6 8 10 12 14 16 18 20 22 24 26

FIGURE 2

Fig. 1 Growth curves of animals in experiment I.
Fig. 2 Growth curves of animals in experiment II.

the choline and pyridoxine had a much more striking effect on the growth rate.

This experiment would seem to indicate that enriched flour did not contain enough riboflavin to support normal growth when included in the diet at a 40% level, as the addition of more riboflavin along with choline and pyridoxine aided in accelerating the growth rate.

Second experiment: Effect of the addition of B vitamins to enriched flour, on growth, maintenance and reproduction

The second experiment was set up on a long term basis to determine the growth rate, maintenance, reproduction and length of life of the animals fed the different diets. Seven diets (table 1) were used with 10 animals on each diet. Diets I and II, containing 40% whole wheat and enriched flour, respectively, and diet VII, the stock ration, were considered the control diets. Diet III had 0.75 mg choline and 2 μg pyridoxine added per gram of enriched flour in order to meet the rat's requirements for these 2 materials. Diet IV had 1 mg of liver extract added per gram of enriched flour in order to furnish all the B vitamins. Diet V had 0.75 mg of choline, 2 μg pyridoxine and 14.2 μg riboflavin added to each gram of enriched flour in order to meet the requirement of the rat for these essentials. Diet VI was like diet III except that 20.5 μg of calcium pantothenate was added per gram of enriched flour.

The average growth rates of the rats on the different diets are shown in figure 2. The animals surviving on diet I barely held their weight for 14 weeks of the test. They were denuded of fur, had a dermatitis on the feet and were inactive. These details are brought out on plate 1, A.

Analysis of the whole wheat showed that it contained 4.69 μg of thiamine, 0.92 μg riboflavin, 5.93 μg of calcium pantothenate and 49.63 μg of nicotinic acid per gram. The animals received 11.26 μg of thiamine, 14.23 μg of calcium pantothenate, 2.21 μg of riboflavin and 119.11 μg of nicotinic acid per day. The diet was low in calcium pantothenate since Supplee, Bender and

Kahlenberg ('42) reported that 20 μg of calcium pantothenate was needed for adequate growth. The riboflavin in the diet was very low, 2.21 μg per day. Inadequate calcium pantothenate and riboflavin undoubtedly accounted for the death of some of the animals on this diet and the loss of fur and failure to gain weight by the others.

At the beginning of the 15th week of the test, 14.2 μg per gram of riboflavin was added to the ground wheat used in diet I in order to bring the riboflavin intake nearer the normal amount required by the rat. All animals gained weight rapidly, as shown in figure 1. Ten weeks after the addition of riboflavin (24th week of the test), these animals had gained considerable weight. Their fur grew again; they looked and acted like normal animals (plate 1, B). They were not so large as those on the normal diet, which had made an average gain of 276 gm during the same period. The 2 females produced young but only 1 litter was raised.

At 3 months of age, 5 females of the second generation on diet I were bred but only 1 produced a litter. There were 10 young but these soon died. Evidently the diet containing whole wheat supplemented with riboflavin was sufficient for growth but not for reproduction and lactation in the second generation.

Diets II through VI contained enriched flour. The flour assayed 7.82 μg of thiamine, 2.69 μg riboflavin, 2.05 μg calcium pantothenate and 42.63 μg of nicotinic acid per gram. The diet provided 18.77 μg of thiamine, 6.46 μg riboflavin, 4.92 μg calcium pantothenate and 102.3 μg nicotinic acid daily.

The animals on diet II, enriched flour, made better weight gains than those on whole wheat. This may in part be explained by the increase in thiamine and riboflavin in this diet. However, the enriched flour furnished less calcium pantothenate and nicotinic acid than the whole wheat. The animals on diet II did not grow at a normal rate (fig. 2). By the end of 14 weeks they had developed a dermatitis on the paws, loss of fur, and curvature of the spine, as is shown on plate 1, C. They were bred but no young were born. As the test contin-

ued the dermal symptoms increased and some of the animals died. After 46 weeks 2 μg per gram of pyridoxine was added to the enriched flour. This amount of pyridoxine added to that already in the flour was considered sufficient for growth and maintenance. Two weeks after this addition the fur began to grow, the dermatitis was cured, and weight was increased, but they failed to reproduce. This failure may be due to a lack of other vitamins.

Animals on diet III, with choline and pyridoxine added, averaged a slightly better growth rate than those on enriched flour alone (fig. 2). They lost part of their fur and showed the symptoms of a lack of calcium pantothenate, with reddish incrustations on the whiskers, head and paws. Three females had litters but destroyed their young soon after birth. This may have been due to a lack of riboflavin and calcium pantothenate.

Liver extract was added in diet IV in order to determine whether it would aid in promoting better growth than the addition of the synthetic vitamins. The animals on this diet made better weight gains than those on whole wheat or enriched flour (fig. 2). The growth was below normal; evidently not enough liver extract was added to provide the necessary essentials lacking in this diet.

The animals on diet V made better weight gains than those on diets I to IV (fig. 2; plate 2, A). However the animals on diet VI showed even greater weight gains (fig. 2). The amount of calcium pantothenate added plus the amount already in the flour brought the daily intake up to 54.12 μg per rat per day in diet VI. This amount should more than take care of the growth requirement. Rats on this diet made the best growth of those on the experimental diets, but the average growth rate was not equal to that of the animals on the stock diet. These rats appeared active and their fur was in good condition (plate 2, B). The lack of riboflavin in this diet may account for the fact that these animals made less growth than those on the stock diet. The animals on diet VI were mated, but no young grew to maturity. This may have been due to

a lack of riboflavin in the diet, or not enough calcium pantothenate or other vitamins to allow for proper lactation.

Diet VII was the stock diet. Animals on this diet grew at a much faster rate than any of the other groups and showed no deficiency symptoms (fig. 2 and plate 2, C). They were able to produce normal litters and raise them to maturity, which would indicate that the diet was adequate.

This experiment was carried on over a 64-week period in order to compare the length of life of the rats on these diets. Seven of the 10 rats on the diet containing whole wheat were dead by the 14th week. Those on the enriched flour lived a longer time but most of them died in or before the 46th week. The addition of choline and pyridoxine in diet III did not seem to have much effect on the lengthening of life, since these animals died at about the same time as those on the enriched flour alone. The addition of choline, pyridoxine and riboflavin, as in diet V, and the addition of choline, pyridoxine and calcium pantothenate, as in diet VI, proved beneficial since 60 and 70%, respectively, of these animals lived for 64 weeks and then were discarded.

Third experiment: Whole wheat and enriched flour at the 40 and 60% levels

In the third experiment it was decided to compare the effect on growth and reproduction of the animals on diets containing whole wheat, whole wheat with added thiamine, enriched flour, and enriched flour with the addition of sufficient B vitamins to meet the rat's requirements. The whole wheat and the enriched flour were included in the diets at the 40 and 60% levels. The 60% level was included since people in some parts of the world use cereals in the diet at this level. A series of animals on the stock diet were also included for the purpose of comparison. The composition of the diets is given in table 1 and the growth curves are shown in figure 3.

The animals with 40% enriched flour in the diet showed better weight gains than those on whole wheat. This is in accordance with an observation of Guerrant and Fardig ('47).

The addition of 4.4 μg of thiamine per gram to the whole wheat provided an approximate intake per rat per day of 22 μg of thiamine, which would be more than an ample supply of this vitamin for growth purposes. The animals with added thiamine in the diet did make better weight gains than those on whole wheat alone, thereby showing that whole wheat at a 40% level in the diet did not provide enough thiamine for normal growth. The animals with thiamine added to the whole wheat did not make quite such good gains throughout the test as those on enriched flour (fig. 3).

At a 60% level in the diet greater gains were made by the animals on enriched flour than by those on whole wheat (fig. 3). These gains were greater in each case than those of the animals with 40% of these constituents in the diet. This would seem to indicate that if the diets were lacking in B-complex vitamins a higher percentage of cereals in them would provide more of these substances.

It will be noted (fig. 3) that whole wheat at a 60% level in the diet promoted only slightly better growth than enriched flour at a 40% level. This may be due to the difference in vitamin content of the diet or a difference in digestibility of these 2 products. In regard to vitamin content, in diet IV with 60% whole wheat the animals received 16.78 μg of thiamine, 3.31 μg riboflavin, 21.41 μg of calcium pantothenate and 178.7 μg of nicotinic acid. Those on diet III with 40% enriched flour received 18.77 μg thiamine, 6.46 μg riboflavin, 4.92 μg calcium pantothenate and 102.3 μg nicotinic acid. The amount of thiamine obtained from diet IV is slightly less than that of diet III, while diet III contained about twice as much riboflavin, and diet IV had about 4 times as much calcium pantothenate as diet III. It may be possible that the increased calcium pantothenate in the whole wheat accounted for the added weight gain and in some way made up for the lack of riboflavin for growth purposes. The rats on diet V with 60% enriched flour obtained approximately 28.15 μg of thiamine, 9.68 μg riboflavin, 7.38 μg calcium pantothenate and 153.47 μg nicotinic acid per day. There was a higher content of thiamine

and riboflavin in diet V than in diet IV, but the calcium pantothenate content of diet IV was greater than that of V. The increased growth of the animals on diet V over that of the rats on diet IV may have been due to a better balance of the vitamins in the former diet. From our observations, when riboflavin is low and calcium pantothenate plentiful in the diet, along with the other vitamins, the growth is approximately that of animals with lowered amounts of calcium pantothenate and a plentiful supply of riboflavin; but even better growth is obtained when these vitamins are in the diet.

The rats on diets I through V were mated but no young were produced. It may be that the diets were lacking in vitamin E, but it would seem that whole wheat would have enough vitamin E in the germ portion to promote reproduction. It is possible that the lack of some of the B-complex vitamins may have been the cause.

At the beginning of the 20th week of the test the animals on diets III, IV and V were each divided into 2 groups. One group had 4% autoclaved yeast added to the diet and the other had 10% autoclaved peanuts added. These products replaced an equivalent amount of sucrose in the diet. It will be noted (fig. 3) that the autoclaved peanuts did not contain materials needed by the rat, as growth was decreased or remained at the same rate. In the case of the addition of autoclaved yeast, growth was accelerated in all cases, which indicated that the yeast contained substances besides thiamine which had a beneficial effect upon growth.

Diet VI had choline, riboflavin, pyridoxine and calcium pantothenate added to the enriched flour while diet VII had these same additions plus 4.4 μg of thiamine per gram. The growth curves indicate that the animals on these 2 diets approached the same rate of growth as those on the stock diet. At the end of the 20th week the average weights were 235, 267, and 281 gm, respectively. Since the animals on diet VII made better gains than those on diet VI, it seems evident that the addition of the thiamine was beneficial. None of the rats

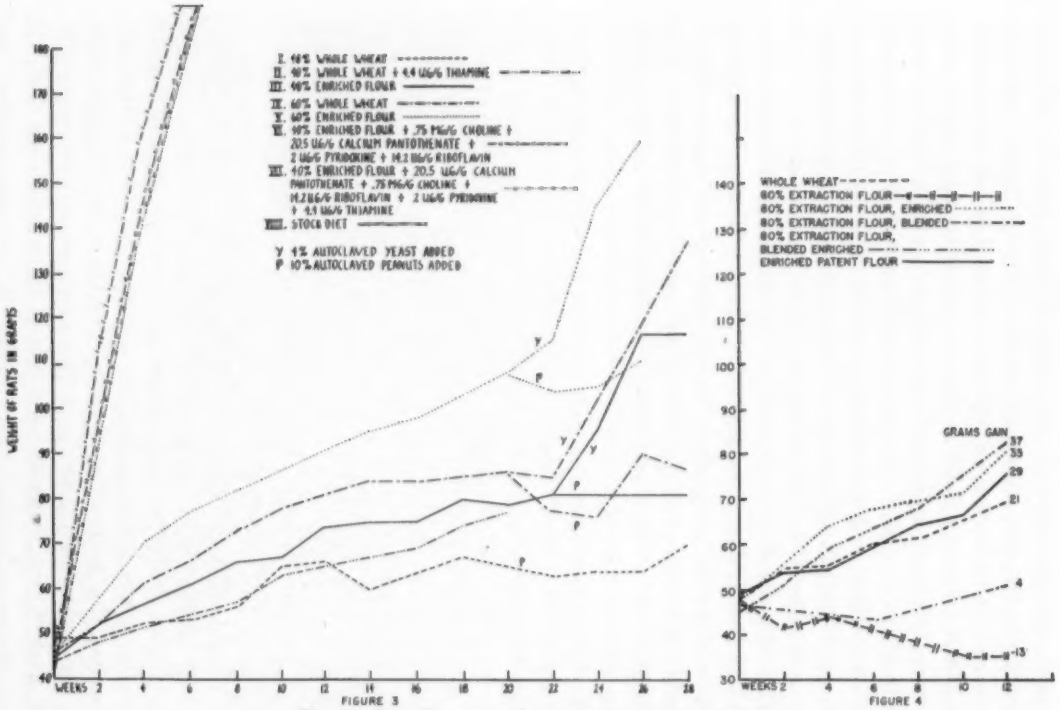
I. 96% WHOLE WHEAT
II. 96% WHOLE WHEAT + 4.4 UG/G THIAMINE
III. 96% ENRICHED FLOUR
IV. 60% WHOLE WHEAT
V. 60% ENRICHED FLOUR
VI. 40% ENRICHED FLOUR + .75 MG/G CHOLINE + 20.5 UG/G CALCIUM PANTOTHENATE + 2 UG/G PYRIDOXINE + 14.2 UG/G RIBOFLAVIN
VII. 40% ENRICHED FLOUR + 20.5 UG/G CALCIUM PANTOTHENATE + .75 MG/G CHOLINE + 14.2 UG/G RIBOFLAVIN + 2 UG/G PYRIDOXINE + 4.4 UG/G THIAMINE
VIII. STOCK DIET
Y 4% AUTOCLAVED YEAST ADDED
P 10% AUTOCLAVED PEANUTS ADDED
WEIGHT OF RATS IN GRAMS
WEEKS
FIGURE 3
WHOLE WHEAT
80% EXTRACTION FLOUR
80% EXTRACTION FLOUR, ENRICHED
80% EXTRACTION FLOUR, BLENDED
80% EXTRACTION FLOUR, BLENDED ENRICHED
ENRICHED PATENT FLOUR
GRAMS GAIN
37
33
29
21
4
-13
WEEKS
FIGURE 4

Fig. 3 Growth curves of animals in experiment III.
Fig. 4 Growth curves of animals in experiment IV.

on the 2 diets made quite so much gain throughout the test as those on the stock diet.

At the beginning of the 14th week of the test, the animals on these diets were mated. Three of the 4 females on diet VI had litters but destroyed the young. This happened again in the second mating which would seem to indicate that the diet was not adequate for raising the young.

Of the rats on diet VII, with thiamine added, 3 out of 4 females produced litters. The young were weaned at the age of 21 days and kept on diet VII for a growth test. Their average growth rate approximated that of the first generation. By the 14th week after weaning, they had an average weight of 216 gm while those of the first generation at the same age weighed 221 gm. The animals were mated at this time and given 3 mg of vitamin E each day to make up for any deficiency of this vitamin in these diets. None of the second generation animals produced live young. The diet was evidently lacking in some other factor necessary for reproduction and lactation.

Fourth experiment: Effect on growth of 80% extraction flour

When the government began the policy of shipping more wheat and flour abroad it became necessary to conserve these materials at home. In order to do this the government made it mandatory that 80% extraction flour be milled in this country for a time. Immediately the question was raised as to whether or not this type of flour would have more nutritive value. An experiment was set up to compare the effect on the growth of the rat when fed a diet containing whole wheat, enriched patent flour, and 80% extraction flour. The whole wheat was taken from the same sample as that used in milling the 80% extraction flour. The enriched patent flour was a part of the same used in the previous experiments, obtained from a commercial mill.

The 80% extraction flour was milled by the Department of Milling Industry of the college. Since this was a change in

the processing, it was suggested that it might be difficult to set the mill to obtain exactly 80% extraction flour; therefore included in the test was an 80% extraction flour which was blended from flour milled at 5 different mills. Also it was decided to include 80% extraction flour which had been enriched to the same level with the B-complex vitamins as the enriched patent flour. It was thought this might show whether or not there were enough of the other B-complex vitamins in the 80% extraction flour to promote as good growth as the enriched patent flour.

To determine the amount of each vitamin needed to enrich the 80% extraction flour, analyses were made of the enriched patent flour and of the 80% extraction flour. The enriched patent flour contained 7.82 μg of thiamine, 3.32 μg of riboflavin and 44.2 μg of nicotinic acid per gram. The 80% extraction flour contained 3.28 μg of thiamine, 0.32 μg of riboflavin, and 10.0 μg of nicotinic acid per gram. Therefore it was necessary to add 4.54 μg of thiamine, 3.00 μg of riboflavin, and 34.2 μg of nicotinic acid per gram to the 80% extraction flour in order to bring the content of these vitamins up to the same levels.

The 6 different diets used in the experiment contained the whole wheat and flour at a 40% level. The entire composition of the diets is given in table 1. The growth tests were conducted over a period of 12 weeks and the results are shown in figure 4. The animals on diet II with the 80% extraction flour without enrichment made no weight gains; instead, they lost 13 gm in weight (fig. 4). Animals of diet IV with 80% extraction flour, blended, also showed very poor growth and gained only 4 gm during the test. The animals on both of these diets showed the B-complex deficiency symptoms of inactivity, loss of appetite, nervousness and in some cases paralysis.

Diet I, containing whole wheat, produced an average weight gain of 21 gm during the test period while the animals on diet VI with enriched flour gained an average of 29 gm. This is in accord with the previous tests where the animals on enriched flour at a 40% level in the diet made better weight gains than those on whole wheat. The rats on diets III and V with the

enriching agents added to the 80% extraction flour showed slightly better weight gains throughout the test than those with enriched flour in the diet; their average gains were 33 and 37 gm, respectively.

The results would seem to indicate that if 80% extraction flour is to be used as a source of B-complex vitamins in the diet, it should be enriched; then it is only slightly better in promoting growth in rats than is the enriched patent flour.

SUMMARY

Experiments have been conducted on albino rats to determine the effect on growth, maintenance, reproduction and length of life of the use of whole wheat, enriched flour, enriched flour supplemented with other B-complex vitamins, and 80% extraction flour in the diets. Under the conditions of the experiments it was found that the whole wheat and the enriched flour did not support normal growth and reproduction in the rats when included in the diet as sources of the B-complex vitamins, at either the 40 or 60% level. However, the enriched flour in the diet at these levels promoted better growth than the whole wheat.

Under the conditions of these experiments the enriched flour supplemented further with the B vitamins supported better growth, maintenance, reproduction and length of life than the enriched flour alone. The addition of choline, pyridoxine and riboflavin to the enriched flour resulted in greater weight gains than the addition of choline and pyridoxine. However, when choline, pyridoxine and calcium pantothenate were added even better growth was obtained. The animals with additional riboflavin or calcium pantothenate in the diet lived longer and showed fewer deficiency symptoms than those on the diet of enriched flour with only choline and pyridoxine added.

Animals on diets with choline, pyridoxine, calcium pantothenate and riboflavin added to the enriched flour, and these vitamins plus thiamine, made excellent growth records which compared favorably with the average gain made by the ani-

mals on the stock diet. None of the animals on the test diets reproduced so well as those on the stock diets.

The 80% extraction flour, without enrichment, did not support growth in the rats. When 80% extraction flour was enriched at the same levels as patent flour it supported slightly better growth than the enriched patent flour.

ACKNOWLEDGMENTS

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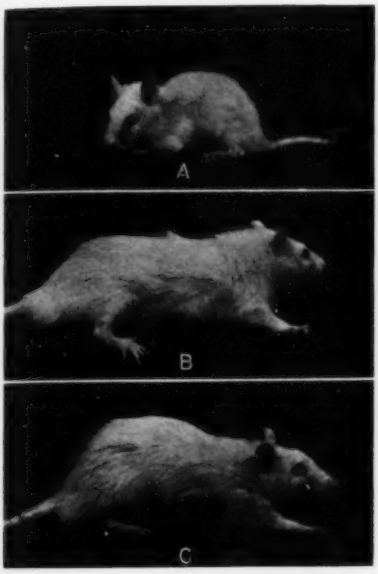

PLATE 1

EXPLANATION OF FIGURES

A. After 14 weeks on diet I containing 40% whole wheat.

B. Improvement shown 10 weeks after addition of riboflavin to diet I.

C. After 24 weeks on diet II containing 40% enriched flour.

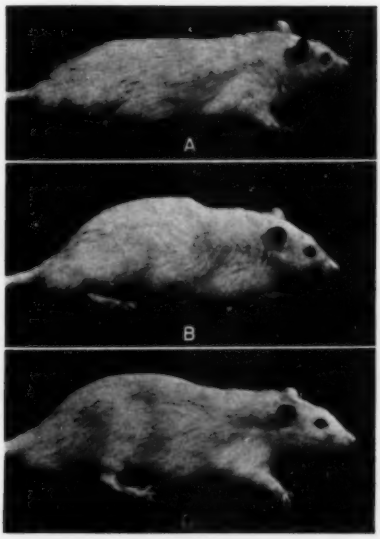

PLATE 2

EXPLANATION OF FIGURES

A. After 24 weeks on diet V containing 40% enriched flour with choline, pyridoxine and riboflavin added.

B. After 24 weeks on diet VI containing 40% enriched flour with choline, pyridoxine and calcium pantothenate added.

C. After 24 weeks on the stock diet.

THE RELATION OF ASCORBIC ACID METABOLISM IN THE RAT TO DIETS HIGH IN PROTEIN, CARBOHYDRATE OR FAT [1]

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Ascorbic acid deficiency has long been associated with disturbances in protein metabolism, particularly the formation of intercellular cementing substance. Sealock and Silberstein ('39) were the first workers to connect this deficiency with the metabolism of specific amino acids. The normal metabolism of certain amino acids is disturbed in scorbutic guinea pigs and immature infants (Levine, Gordon and Marples, '41; Christensen and Lynch, '48). Administration of ascorbic acid, often in large amounts, restores normal metabolism. In the present experiments we have attempted to determine whether ascorbic acid metabolism can be influenced in the rat by increasing protein metabolism while maintaining caloric intake constant.

Rats, therefore, were fed diets high in carbohydrate, fat or protein but in all cases adequate to maintain nitrogen balance, and the ascorbic acid concentrations in various organs determined.

METHODS

Adult male rats of the Sprague-Dawley strain were fed for 5 weeks by the stomach tube technique of Reinecke, Ball and Samuels ('39). The composition of the diets used is given in table 1.

[1] This investigation was supported by grants from the Sugar Research Foundation and from the Medical Research Fund, University of Utah.

In addition, in experiment 2 the protein-fed rats were given 6 mg ascorbic acid per day. In experiment 3 the fat group and 1 protein group received 50 mg ascorbic acid per day by mouth.

At the end of the feeding period the rats were anesthetized with sodium amytal given intraperitoneally, 9 mg/100 gm

TABLE 1

Diets used in stomach tube feeding

	HIGH FAT	HIGH CARBOHYDRATE	HIGH PROTEIN
Lactalbumin, Labco (gm)	30	30	263.25
Gelatin, U.S.P. (gm)	30	30	30
Corn oil (gm)	105.8	3	3
Dextrin (gm)	0	233.25	0
Salt mixture, U.S.P. no. 1 (gm)	12.0	12.0	12.0
Oleum Percomorphum, reinforced (drops)[1]	12	12	12
Vitamin concentrate (gm)[2]	6.0	6.0	6.0
Water to make (ml)	900	900	900
Calories (per ml)	1.30	1.31	1.30
Caloric distribution (% of total calories)			
Protein	19.5	19.5	92.4
Fat	80	2.7	2.7
Carbohydrate	0.5	77.8	4.9

[1] Mead Johnson and Co.

[2] Lederle's Lederplex. According to label, 6 gm contained (in milligrams) thiamine hydrochloride 24, riboflavin 24, nicotinamide 120, pyridoxine 2.4, calcium pantothenate 36, choline 240, inositol 120 and folic acid 2.4; plus other water-soluble extractives from 96 gm liver.

body weight. The abdomen was then opened and blood withdrawn from the abdominal aorta. The various organs were removed, weighed, ground with sand and 6% trichloracetic acid and centrifuged. The supernatant solutions and blood plasma were analyzed for ascorbic acid by the method of Roe and Kuether ('43).

A sample of liver was also analyzed for total nitrogen by the Kjeldahl method.

RESULTS

As can be seen in table 2, there were no significant differences in the ascorbic acid concentrations of any of the tissues when the fat-fed and carbohydrate-fed groups were compared, with the possible exception of the livers in experiment 1. In this case, when ascorbic acid was related to the nitrogen content of the livers, this difference was eliminated. Apparently a wide shift in the ratio of carbohydrate to fat as sources of calories did not affect ascorbic acid metabolism in a marked way.

On the other hand, the protein-fed groups showed significant differences from those on the other 2 diets. The ascorbic acid concentrations of muscle and blood were always lower in the animals receiving the high protein diet than in the other groups. The administration of high doses of ascorbic acid did not alter blood levels significantly, but the concentration in the muscles was definitely increased.

The kidneys and livers of protein-fed rats also had lower concentrations of ascorbic acid on the unsupplemented diets. Both organs, however, were larger in the animals on the high protein diets. When considered on the basis of ascorbic acid per 100 gm body weight, there was as much in the livers of the protein-fed rats as in the carbohydrate- or fat-fed groups; the lower concentration in the liver was offset by the larger size of the organ. This was not true in the case of the kidney; the concentration per 100 gm body weight was also significantly lower unless ascorbic acid was administered. The levels in both kidneys and liver were raised when the vitamin was fed.

As shown in table 3, the significant differences in total weight of organs are those which have been previously reported (Osborne, Mendel, Park and Winternitz, '25). The livers of the rats fed high protein were largest in experiment 2 where the caloric intake was also largest. In experiment 1, the livers of the fat-fed and protein-fed rats were larger than those of the rats fed carbohydrate but did not differ

TABLE 2

Ascorbic acid content of organs of male rats on high fat, high carbohydrate or high protein diets[1]

EXPERIMENT AND DIET	NO. RATS	LIVER			KIDNEY		BRAIN		ADRENALS		BLOOD	MUSCLE
		mg per gm Organ	mg per gm N	mg per 100 gm BW[2]	mg per gm Organ	mg per 100 gm BW[2]	mg per gm Organ	mg per 100 gm BW[2]	mg per gm Organ	mg per 100 gm BW[2]	mg per 100 ml	mg per gm Tissue
Experiment 1												
Fat diet	10	0.242	7.84	0.848	0.153	0.102	0.252	0.145	3.19	0.054	0.90	0.037
		± .017	± 0.71	± .052	± .006	± .005	± .010	± .005	± .22	± .0053	± .04	± .0019
Carbohydrate diet	10	0.262	7.47	0.723	0.130	0.084	0.228	0.137	3.21	0.052	0.94	0.035
		± .014	± 0.53	± .039	± .010	± .005	± .019	± .011	± .14	± .0037	± .06	± .0013
Protein diet	8	0.188	5.27	0.717	0.068	0.064	0.227	0.133	3.19	0.069	0.79	0.026
		± .010	± 0.20	± .047	± .004	± .004	± .013	± .008	± .18	± .0030	± .05	± .0010
Experiment 2												
Fat diet	5	0.289		0.891	0.137	0.091	0.368	0.220	3.86	0.089	0.83	0.025
		± .001		± .022	± .005	± .004	± .018	± .011	± .11	± .0048	± .06	± .0003
Carbohydrate diet	7	0.337		0.883	0.143	0.092	0.380	0.232	3.31	0.064	0.83	0.025
		± .013		± .052	± .006	± .004	± .012	± .011	± .20	± .0055	± .06	± .0008
Protein diet	5	0.222		0.794	0.088	0.085	0.334	0.172	3.89	0.079	0.65	0.019
		± .011		± .048	± .004	± .005	± .008	± .008	± .13	± .0060	± .07	± .0006
Protein diet + 6 mg ascorbic acid injected	6	0.237		0.928	0.092	0.092	0.326	0.183	4.06	0.083	0.64	0.023
		± .024		± .091	± .007	± .004	± .022	± .010	± .13	± .0033	± .05	± .0018
Experiment 3												
Fat diet + 50 mg ascorbic acid fed	7	0.331		0.882	0.221	0.134	0.323	0.195	3.79	0.072	0.96	0.031
		± .008		± .042	± .006	± .006	± .007	± .008	± .18	± .0031	± .04	± .0018
Protein diet	7	0.278		0.792	0.131	0.101	0.308	0.180	3.57	0.062	0.76	0.024
		± .016		± .038	± .003	± .002	± .014	± .011	± .18	± .0046	± .06	± .0010
Protein diet + 50 mg ascorbic acid fed	8	0.303		0.808	0.157	0.124	0.310	0.180	3.86	0.064	0.80	0.028
		± .015		± .040	± .007	± .005	± .006	± .005	± .16	± .0026	± .04	± .0007

[1] All values are given as means with the standard errors of the means.
[2] Total body weight of animal at autopsy.

[2] Total body weight of animal at autopsy.

TABLE 3

Weights of organs of rats on high fat, high carbohydrate, or high protein diets fed by stomach tube

DIET	RATS	CALORIES PER DAY	AVE. WT. AT START	CAL./100 GM/DAY	AVE. WT. AT END	LIVER [1]		KIDNEYS [1]	ADRENALS	BRAIN	THYMUS
						gm	*% N*	*gm*	*gm*	*gm*	*gm*
Experiment 1											
Fat diet	10	50.1	252	19.9	299	10.62 ± .46	3.30 ± .05	2.016 ± .081	0.050	1.726	0.214
Carbohydrate diet	10	48.0	249	19.3	285	7.91 ± .26	3.58 ± .05	1.878 ± .065	0.044	1.712	0.285
Protein diet	8	48.0	247	19.4	282	10.07 ± .30	3.72 ± .06	2.636 ± .101	0.050	1.676	0.211
Experiment 2											
Fat diet	5	48.0	296	16.2	291	8.97 ± .32	3.28 ± .20	1.944 ± .034	0.067	1.748	
Carbohydrate diet	7	48.0	295	16.3	292	7.61 ± .18	3.55 ± .03	1.884 ± .193	0.055	1.801	
Protein diet	5	55.0	304	18.1	338	12.14 ± .62	3.59 ± .03	3.269 ± .139	0.069	1.731	
Prot. and 6 mg ascorbic acid injected	6	52.0	286	18.2	318	12.28 ± .42	3.54 ± .06	3.183 ± .144	0.062	1.771	
Experiment 3											
Fat + 50 mg ascorbic acid fed	7	48.0	297	16.2	289	7.66 ± .29	3.59 ± .07	1.740 ± .053	0.056	1.732	
Protein diet	7	48.0	298	16.1	293	8.37 ± .37	3.84 ± .02	2.270 ± .077	0.050	1.700	
Protein + 50 mg ascorbic acid fed	8	48.0	308	15.6	296	7.97 ± .18	3.80 ± .04	2.364 ± .037	0.050	1.752	

[1] Values are given as means with the standard errors of the means.

significantly between themselves. However, the nitrogen content of the livers of the protein-fed groups was always higher than that in the other groups of all 3 experiments. In experiment 1 where all rats received equicaloric rations in quantities sufficient to cause gains in weight, the rats receiving fat had accumulated enough lipid in the liver to offset the increased protein content of the livers of the protein-fed rats. In experiment 3 where the animals were slightly underfed, the livers tended to be smaller and to have higher nitrogen contents, but the difference between diets was still present.

The kidneys of the protein-fed rats were always larger. The difference was more marked if the calories on the high protein diet were sufficient for gains in weight, but it was still marked in experiment 3 where food intake was insufficient to maintain weight.

There was no significant difference in the size of the adrenals of the protein- and fat-fed rats. The average weight of the adrenals of the carbohydrate-fed animals was slightly smaller. These results confirm the observations of Ingle, Ginther and Nezamis ('43) that, in the adult rat, there is no significant increase in adrenal size on a protein diet. Since in these experiments 92% of the calories was obtained from protein it seems unlikely that the adrenals play any role in the catabolism of dietary protein; otherwise, some evidence of hypertrophy would have been expected.

DISCUSSION

There seems to be some effect of protein diet on the metabolism of ascorbic acid in the rat. While there is no definite evidence of the mechanism involved, it seems that the following hypothesis would most simply fit the facts:

As Sealock, Perkinson and Silberstein ('40) have shown, ascorbic acid is related to the metabolism of tyrosine and phenylalanine. It also appears to be involved in the metabolism of glutamine and glycine (Christensen and Lynch, '48). On the high protein diet used here relatively large amounts of these amino acids, as well as all others, were catabolized.

In such a situation it might be expected that the utilization of ascorbic acid would be greatly increased. Production, in which the liver appears to play a prominent role (Smythe and King, '42), could not keep up with destruction, and concentrations in the plasma and in the tissues which would metabolize such amino acids decreased. In those tissues like the brain, which did not use these amino acids, or in those tissues where ascorbic acid appears to be associated with some other specific metabolic process, such as the adrenals, there was no significant effect of the diet.

When ascorbic acid was fed, the concentrations in the tissues where they were low were increased, but the vitamin was still removed so rapidly by these tissues that the plasma level did not rise significantly. This would be expected, since the distribution of ascorbic acid to the tissues is not simply a diffusion phenomenon, but must involve considerable energy utilization to maintain the marked concentration gradient. If there was an increase in the processes utilizing ascorbic acid it might be expected that there would be energy available for maintaining an increased concentration gradient. In no single tissue did this gradient reach the maximum for that tissue found by Kuether, Telford and Roe ('44) in guinea pigs.

On the other hand, if decreased production were primarily responsible for the low ascorbic acid values, one would have expected that the levels in all tissues, whether they utilized amino acids or not, would be decreased. This is seen in the scorbutic guinea pig where the levels in the brain and adrenals follow the general drop in tissue concentration. One would also have expected the circulating fluids to show the effect of dietary ascorbic acid, if tissue demand were normal and liver production alone decreased. Also, as Roberts and Spiegl '47) demonstrated, the production of ascorbic acid, like other functions of the liver, is dependent on the supply of sulphur-containing amino acids. These were not lacking in any of the diets, and certainly not in the high protein ration. A relationship between tyrosine catabolism and ascorbic acid has been

demonstrated in liver and kidney tissues by Lan and Sealock ('44). It probably is also true for muscle since the aromatic amino acids undergo metabolism there. It seems most logical, therefore, to assume that the changes observed were due to increased destruction of ascorbic acid in the presence of high amino acid metabolism.

Heinemann ('36) studied the influence of a diet containing 50% of the calories as protein on the excretion of, and requirement for, ascorbic acid in the human being. A diet containing 11% of the calories as protein was used as the basis of comparison. He found no effect. Since our high protein diets contained 92% of the calories as protein the apparent difference in results may be due to the greater sustained protein level in our experiments. It may also be due to the different methods of study or to difference in species. Certainly there seems to be an influence of high protein diets on the tissue distribution of ascorbic acid in the rat.

CONCLUSIONS

A high protein diet led to a decrease in the concentration of ascorbic acid in tissues which metabolize considerable amounts of amino acids, such as the liver, kidneys and muscle. The levels in the plasma were also decreased.

There was no change in brain or adrenal ascorbic acid concentrations.

The enlargement of the liver on a high protein diet was sufficient to leave no significant difference in the amounts of liver ascorbic acid per unit body weight. This was not true of the kidney. This organ showed a marked increase in size on the high protein diet, but the concentrations of ascorbic acid were so low that there was less ascorbate per unit of body weight.

Administration of ascorbic acid led to an increase in the concentration of ascorbate in the liver, kidneys and muscle of protein-fed rats, but even doses of 50 mg a day did not significantly increase the concentration in the plasma.

It is thought that the results are best explained by an increased utilization of ascorbic acid in those tissues able to

metabolize large amounts of amino acids. The muscle illustrates the changes in a tissue metabolizing both ascorbate and protein, the blood reflects the body balance, and the liver shows the compensation changes of a producing organ.

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THE NEED FOR AND INTERRELATIONSHIP OF FOLIC ACID, ANTI-PERNICIOUS ANEMIA LIVER EXTRACT, AND BIOTIN IN THE PIG

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ONE FIGURE

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Available literature on attempted experimental production of anemia in animals was reviewed in an article by Miller and Rhoads ('35). These workers found that feeding a modified canine-black-tongue-producing diet to swine produced a symptom-complex marked by oral mucus membrane lesions, achlorhydria, and anemia. There was remission of the anemia and amelioration of symptoms as a result of oral or parenteral administration of liver extract.

Cartwright, Wintrobe and Humphreys ('46) using a purified diet containing 2% sulfasuxidine in the ration, produced in a pig an anemia which responded to a highly purified liver extract but not to biotin. They suggested that in the pig a "folic acid" deficiency may be associated with the develop-

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ment of a normocytic anemia. In the rat, a similar dietary regimen produced a deficiency in growth that responded promptly to a combination of biotin and pteroylglutamic acid and showed partial response to a 1% level of either yeast concentrate or liver extract (Welch and Wright, '43).

Welch et al. ('47) by use of a crude chemical antagonist that interfered with the metabolism of pteroylglutamic acid in the pig, interrupted and significantly inhibited the formation of erythrocytes and granulocytes. The addition of a crude source of extrinsic factor, together with normal human gastric juice, was found to alleviate the interference.

Cunha et al. ('47) failed to produce an anemia in swine by use of a purified diet including sulfathalidine for a 5-week period at a 0.5% level supplemented with 6 B-complex vitamins. They found that the addition of "folic acid" or p-aminobenzoic acid (PABA) alone to the basal purified ration stimulated hemoglobin formation to a small extent. However, when folic acid was added to the purified basal ration in addition to an anti-pernicious anemia liver extract or other vitamins (inositol, biotin, PABA), it did not stimulate hemoglobin formation, possibly indicating some vitamin imbalance.

The experiment reported herein gives data on the production of a folic acid deficiency with the pig and on an interrelationship of pteroylglutamic acid and an anti-pernicious anemia liver extract with biotin. In addition, folic acid and the anti-pernicious anemia liver extract are compared in preventing an anemia which developed in the pig.

EXPERIMENTAL

Six-week-old purebred Chester White pigs were used in this experiment. They were divided equally in each lot as to condition, weight, sex, and age.

The rations fed are shown in tables 1 and 2. The same pigs were used throughout the 2 phases of this experiment. During the first phase (13 weeks) the effect of sulfasuxidine on the need for folic acid by the pig was studied. Also, the effect of

using a liver preparation,[4] and the addition of folic acid alone and in combination with biotin and 10% of dehydrated alfalfa meal to the basal ration was investigated. When alfalfa meal was added to the basal ration the amount of casein was lowered so that the total protein content of the ration was the same

TABLE 1

The effect of sulfasuxidine (S) on the folic acid (FA), anti-pernicious anemia liver extract (APLE), and biotin (B) needs of the pig

RATION FED	BASAL ONLY [1]	BASAL + S [2]	BASAL + S + FA [3]	BASAL + S + APLE [4]	BASAL + S + FA + B [5]	BASAL + 10% ALFALFA
Lot number	I	II	III	IV	V	VI
Number of pigs [6]	3	3	4	4	4	4
Av. daily gain (lbs.)	0.72	0.61	0.65	0.67	0.71	0.70
Av. daily feed consumption (lbs.)	1.34	1.34	1.35	1.37	1.36	1.36
Lbs. of feed per lb. of gain	1.87	2.20	2.10	2.04	1.91	1.96

[1] Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline).

[2] Sulfasuxidine fed at a level of 2% of the ration.

[3] Crystalline folic acid was fed in both lots at a level of 50 μg per 100 gm of feed.

[4] Injected intramuscularly, in the gluteal muscle, every second day. It was injected at the rate of 0.1 ml daily (1 U.S.P. injectable unit).

[5] Biotin was fed at a level of 20 μg per 100 gm of feed.

[6] These pigs were 6 weeks old and averaged approximately 24 lbs. in weight at initiation of this 13-week trial.

as the basal ration. During the second phase (8 weeks) the effect of a crude folic acid antagonist [5] preparation on the need for folic acid by the pig was studied.

The purified basal ration used in this experiment was the same as that reported previously by Heinemann, Ensminger,

[4] "Refined Solution Liver Extract, Parenteral" used for treatment of human pernicious anemia. Made from pork. Supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

[5] The crude folic acid antagonist (batch N67) was obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

TABLE 2

The effect of sulfasuxidine (S) and crude folic acid antagonist (ANT) on the folic acid (FA), anti-pernicious anemia liver extract (APLE), and biotin (B) needs of the pig

RATION FED	BASAL ONLY [1]	BASAL + S [2]	BASAL + S + ANT [3]	BASAL + S + FA [4]	BASAL + S + ANT + FA [5]	BASAL + S + APLE [6]	BASAL + S + ANT + APLE [7]	BASAL + S + FA + B [8]	BASAL + S + ANT + FA + B
Lot number	I	IIA	IIB	IIIA	IIIB	IVA	IVB	VA	VB
Number of pigs [9]	3	1	2	2	2	2	1	2	2
Av. initial wt. (lbs.)	88.3	89	73	94	73	84	93	95	83
Av. daily gain (lbs.)	0.66	0.41	0.44	0.39	—0.19	0.23	0.04	0.63	0.49
Av. daily feed consumption (lbs.)	2.04	2.01	1.78	1.96	1.27	1.86	1.39	2.06	1.77
Lbs. of feed per lb. gain	3.09	4.89	4.07	4.99	...	8.00	38.93	3.25	3.60

[1] Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline).

[2] Sulfasuxidine was fed at a level of 2% of the ration.

[3] Crude folic acid antagonist was fed at a level of 0.2% of the ration.

[4] Crystalline folic acid (no antagonist fed) was given at a level of 50 μg per 100 gm of feed.

[5] Crystalline folic acid (when antagonist was fed) was given at a level of 100 μg per 100 gm of feed.

[6] Injected intramuscularly, in the gluteal muscle, every second day. It was injected at the rate of 0.1 ml daily (no antagonist fed) (1 U.S.P. injectable unit).

[7] Injected intramuscularly, in the gluteal muscle, every second day. It was injected at the rate of 0.2 ml daily (when antagonist was fed) (2 U.S.P. injectable units).

[8] Biotin was fed at a level of 20 μg per 100 gm of feed.

[9] These pigs were 19 weeks old at the initiation of this 8-week trial.

Cunha and McCulloch ('46). The ration consisted of casein [6] 26.1%, sucrose 57.7%, lard 11%, and mineral mix [7] 5.2%. Water soluble vitamins were fed (in mg per kg of live weight per day) as follows: thiamine 0.52, riboflavin 0.12, niacin 1.20, pantothenic acid 0.50, pyridoxine 0.20, and choline chloride 10.00; fat soluble vitamins were supplied (per pig daily) as follows: vitamin A, 5,000 I.U., vitamin E, 57 mg, vitamin K, 2 mg, vitamin C, 50 mg, and vitamin D, 700 I.U.

A modification of the "paired feeding technique" (Mitchell and Beadles, '30) was used in order to maintain the same feed intake by all pigs fed purified rations. Thus, the feed intake of the pigs was limited to the amount consumed by the pig with the least appetite — with the vitamins and the sulfonamide studied being the only variables among the lots of pigs. Later in the experiment as the appetite varied so much between lots of pigs, the paired feeding technique was altered. The pigs were fed in individual feeding stalls. To prevent coprophagy the pigs were kept on raised floors which were washed twice daily. At no time during the experiment was any evidence of coprophagy observed.

To prevent rancidity and subsequent destruction of vitamins, the purified ration was mixed every third day and kept in an ice box. All vitamin solutions were stored under refrigeration. The required amount of the vitamins fed each pig was measured in calibrated pipettes and placed on top of the ration every other day (every fourth feeding) just before feeding time.

Each pig was bled weekly from the marginal ear vein. The blood examined was for hemoglobin, erythrocyte, leucocyte, and differential cell counts as well as reticulocytes.

Pipettes were filled from the flowing blood on the ear. Smears were prepared from the same source, the stagnant blood being wiped away as it gathered. Hemoglobin was determined according to the method of Evelyn ('36). Leuco-

[6] Vitamin Test Casein GBI, manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio. Alcohol extracted.

[7] Same as used by Wintrobe ('39), and as modified by Heinemann et al. ('46).

cytes were counted in 2% acetic acid diluent tinted with gentian violet. Erythrocytes were diluted with Hayem's solution. Smears from differential leucocyte counts were stained with Wright's stain and 200 cells were examined. Reticulocyte smears were stained with brilliant cresyl blue and counter-stained with Wright's stain.

RESULTS AND DISCUSSION

The data in table 1, during the first 13-week trial, show that no beneficial effect on growth or efficiency of feed utilization was obtained when the anti-pernicious anemia liver extract, or folic acid alone and in combination with biotin, was added to a purified ration containing sulfasuxidine. The addition of 10% dehydrated alfalfa meal to the purified basal ration resulted in no beneficial effect on growth or efficiency of feed utilization.

In lots III and IV where folic acid and the anti-pernicious anemia liver extract were fed, biotin deficiency symptoms showed up 2 weeks earlier (after 5 weeks on experiment) than in lot II where the pigs were fed the basal ration plus sulfasuxidine. The biotin deficiency symptoms obtained were similar to those described by Lindley and Cunha ('46) and Cunha et al. ('46). The pigs in lots III and IV developed much more severe biotin deficiency symptoms than the pigs in lot II. Apparently, the addition of either folic acid or the anti-pernicious anemia liver extract to the basal ration plus sulfasuxidine caused biotin deficiency symptoms to appear sooner and to develop much more severely (fig. 1). This may have been caused by some vitamin imbalance. Suggestions of a vitamin imbalance were obtained by Cunha et al. with rats ('43), with sows ('44), and young pigs ('47), and by Richards with rats ('45). No biotin deficiency symptoms were obtained in lots I, V, and VI.

In both phases of this experiment, occasional scouring, characterized by grayish feces, was obtained in all lots of pigs fed the purified ration except for the pigs fed the basal ration + 10% alfalfa which was fed only during the first phase. The

scouring usually lasted from 36 to 48 hours and did not seem to have any effect on appetite. Similar scours were prevented last year by including sulfathalidine in the ration at a 0.5% level (Cunha et al., '47). However, sulfasuxidine at the level fed in this experiment did not prevent the scouring which occasionally occurs with the basal ration used.

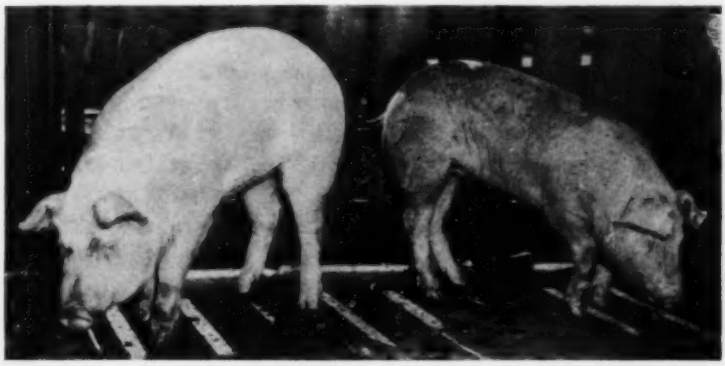

Fig. 1 During the first 13 weeks on experiment, the pig on the right was fed the basal ration + sulfasuxidine + folic acid. The pig on the left was fed the same ration but without folic acid. This picture illustrates the effect of folic acid in increasing the pig's need for biotin as shown by the pig on the right exhibiting severe biotin deficiency symptoms. Similar results in increasing biotin needs for the pig were obtained when an anti-pernicious anemia liver extract was added to the basal ration + sulfasuxidine.

The data given in table 2 show the results obtained during the second phase of the experiment which lasted for 8 weeks. The detrimental effect of feeding sulfasuxidine over a long period of time is evidenced by the decreased growth rate and increased feed required per pound of gain. The addition of a crude folic acid antagonist preparation to the basal ration plus sulfasuxidine did not lower gains or efficiency of feed utilization as compared with the feeding of sulfasuxidine alone. However, the addition of a crude folic acid antagonist preparation to the pigs fed the same ration (basal + sulfasuxidine + folic acid — lot IIIB), the anti-pernicious anemia

liver preparation (lot IVB) and folic acid plus biotin (lot VB) proved to be quite effective in decreasing growth and efficiency of feed utilization. The pigs in lot I (basal) maintained approximately the same average daily gains throughout both phases of the experiment. During the second phase of this experiment the average daily gain of the pigs in lot IIA (basal + sulfasuxidine) was lower than that of the first phase by 0.20 pound. One pig in lot IIIB died 21 days after being fed the crude folic acid antagonist. On necropsy the pig showed the following gross pathology: spleen slightly enlarged, some renal hyperemia, and very extensive bronchopneumonia. The average daily weight loss of this lot of pigs was 0.19 pound. One pig in lot IVB died 3 days after being fed the folic acid antagonist. On necropsy this pig showed the following gross pathology: gross hemorrhage (whole blood) in both lungs, and peripheral infiltration of blood in spleen with swelling. The other pig in this lot gained only 2 pounds during the 8 weeks on this trial, or made an average daily gain of only 0.04 pound. It is difficult to state whether or not the deaths were due to the folic acid antagonist. However, the 2 pigs which died were the only death losses occurring during the experiment. Some deaths were reported in rats by Franklin et al. ('47) when this same crude folic acid antagonist was fed and insufficient folic acid was given.

In lot VA (basal + sulfasuxidine + folic acid + biotin) the gains were approximately equal to those of the pigs on the basal ration, showing that a combination of folic acid and biotin was effective in making up the decrease in growth caused by feeding sulfasuxidine in the ration, a finding in line with similar work obtained with the rat by Nielsen and Elvehjem ('42). The pigs in lot VB fed the same ration plus the folic acid antagonist gained at a slower rate than the pigs in lot VA. However, their average daily gains were greater than those of all lots of pigs except lots I and VA.

The biotin deficiency symptoms became much more pronounced during this second phase of the trial. The pigs in lot I did not, however, display any signs of a biotin deficiency.

Their hair coat was smooth and there were no cracks in their feet. The pig in lot IIA (basal + sulfasuxidine) developed some cracks in his feet and there was some hair loss on the lower and posterior hams. This showed that feeding sulfasuxidine over a long period of time will result in the appearance of slight symptoms of biotin deficiency. The pigs in lot IIB (basal + sulfasuxidine + antagonist) had only slight cracks in their feet, but their hair loss was slightly more pronounced with a dark exudate on the skin of 1 of these pigs. The pigs in lot IIIA (basal + sulfasuxidine + folic acid) displayed very severe biotin deficiency symptoms. Their feet were badly cracked and their hair coat was quite rough, the hair being sparse over the body and none was left on the hams. The pigs also had a small amount of brownish exudate throughout the surface of their body. The 1 pig still alive at the end of this trial in lot IIIB (basal + sulfasuxidine + folic acid + antagonist) had such badly cracked feet that it could hardly walk. The feet bled quite easily. The hair coat was rough, sparse and thin on the back and lower hams and the skin was covered with a dark exudate. The pigs in lot IVA (basal + sulfasuxidine + anti-pernicious anemia liver extract) also had badly cracked feet and walked with difficulty. Their hair coat was also rough. The hair on 1 pig was quite thin all over the body. Both pigs in this lot had a dark exudate on their skins. The 1 surviving pig in lot IVB (basal + sulfasuxidine + anti-pernicious anemia liver extract + antagonist) also had badly cracked feet. The results obtained during the second phase of this trial are in line with those in the first trial where the addition to the basal ration + sulfasuxidine of either folic acid or the anti-pernicious anemia liver extract increased the pigs' needs for biotin since the pigs exhibited very much more severe biotin deficiency symptoms. This is of considerable interest since it may suggest a possible need for biotin by human patients treated with folic acid or the anti-pernicious anemia liver extract. This statement takes on added significance due to the report of Heinle and Welch ('47) suggesting that the possibility needs to be considered that folic acid not

only fails to prevent but actually may precipitate neurologic relapse in some patients with pernicious anemia. At this station a spasticity of the hind legs has continually been observed in pigs with a biotin deficiency.

STUDIES OF THE BLOOD

The data contained in table 3 summarizes the results obtained in the blood studies with pigs during both phases of the experiment.

The data show that the basal ration, after being fed for 21 weeks, is not adequate for hemopoiesis in the pig. Red blood cell formation was more adversely affected than hemoglobin formation. A slight hyperchromic anemia developed. On the basis of these data and those obtained by Cunha et al. ('47) where folic acid stimulated hemoglobin formation with the same basal ration, it is recommended that folic acid be added to the basal ration containing the 6 B-complex vitamins. Of interest is the finding that adding alfalfa to the basal ration stimulated hemopoiesis to about the same extent as folic acid. Since alfalfa is a good source of folic acid, it may be that a good deal or all of the response is due to the folic acid in this plant.

The addition of sulfasuxidine to the basal ration for 21 weeks resulted in a moderate anemia being produced. Folic acid was very effective in preventing this anemia. It was more effective than the anti-pernicious anemia liver extract in preventing the anemia and in stimulating hemoglobin and red blood cell formation when sulfasuxidine alone was added to the basal ration. However, it must be realized that only 1 level of the anti-pernicious anemia liver extract was tried and that possibly a higher level may have given different results. A combination of folic acid and biotin was no better than folic acid alone in stimulating hemoglobin and red blood cell formation when sulfasuxidine was added to the basal ration.

The addition of a crude folic acid antagonist to the basal ration + sulfasuxidine resulted in a more severe anemia be-

TABLE 3

Blood picture of the pigs: hemoglobin (HB), erythrocytes (RBC) and leucocytes (WBC)

LOT NO.	RATION	AV. HB [1] First phase (13 wks.)	AV. HB [1] Second phase (8 wks.)	HB Low point during second phase	RBC Low point during second phase	AV. RBC [1] Second phase (8 wks.)	AV. RBC [1] First phase (13 wks.)	AV. WBC [1] First phase (13 wks.)	AV. WBC [1] Second phase (8 wks.)	COLOR INDEX [2] Assuming 12.5 gm Hb. and 7,000,000 RBC as being near normal
		gm	*gm*	*gm*	*Millions*	*Millions*	*Millions*	*Thousands*	*Thousands*	
I	Basal	10.80	10.88	9.57	4.76	4.69	5.66	20.0	23.4	1.12
II	Basal + sulfasuxidine	10.63	8.60	7.52	4.09	4.18	5.66	18.3	15.3	1.04
	IIB: same as II + Antagonist		8.50	5.77	3.16	3.88			16.0	1.01
III	Basal + sulfasuxidine + folic acid	12.71	14.42	13.04	7.21	7.95	7.50	22.0	24.4	1.00
	IIIB: same as III + Antagonist		8.86	6.52	4.16	4.78			32.0	0.90
IV	Basal + sulfasuxidine + anti-pernicious anemia liver extract	11.57	10.91	10.52	5.27	5.81	6.01	22.3	18.5	1.12
	IVB: same as IV + Antagonist		8.86	4.18	3.20	4.13			22.6	0.73
V	Basal + sulfasuxidine + folic acid + biotin	12.96	13.62	12.26	6.33	6.92	7.41	19.7	17.8	1.04
	VB: same as V + Antagonist		11.18	9.80	5.79	5.87			25.5	0.95
VI	Basal + 10% alfalfa meal	12.87					6.49	19.8		1.12

[1] Average figures indicate an average value obtained from all determinations conducted weekly.

[2] The color index is based on the low point in Hb and RBC during second phase of experiment, except in lot VI where the data from the first phase were used.

ing produced than when the sulfasuxidine alone was added to the basal ration. Folic acid was only slightly effective in preventing the effect of the antagonist, probably because it was not fed at a high enough level. When folic acid was fed in high enough levels it was able to counteract the effect of the same crude folic acid antagonist with the rat as shown by Franklin et al. ('47). The anti-pernicious anemia liver extract was not as effective as folic acid (at the level both were fed) in stimulating hemoglobin and red blood cell formation when the crude folic acid antagonist was added to the basal ration + sulfasuxidine. Of interest is the finding that a combination of biotin and folic acid was more effective than folic acid alone in stimulating red blood cell and hemoglobin formation when the crude folic acid antagonist was fed.

The number of red blood cells closely followed the level of hemoglobin in the blood. A normocytic anemia was produced in the pig which was prevented by folic acid, and to a lesser extent by an anti-pernicious anemia liver extract when sulfasuxidine was added to the basal ration. This is similar to the type of anemia produced by Cartwright et al. ('46) with the pig fed sulfasuxidine, which was cured with a liver extract. Differences in diets fed and the fact that it takes a long time for sulfasuxidine feeding to result in the development of anemia in the pig may account for the finding of Welch et al. ('47) that they were unable, by feeding sulfasuxidine to the pig, to produce an anemia, such as is reported in this paper and previously by Cartwright et al. ('46).

No leukopenia or decrease in granulocytes below normal occurred by adding sulfasuxidine or the crude folic acid antagonist to the basal ration. All pigs in lots III and IV showed some increase in granulocytes above the level obtained with the pigs in lot II. However, all the pigs in lot V showed very nearly the same level of granulocytes as occurred with the pigs in lot II. Where the granulocytes increased in the pigs in lots III and IV, there was a slight decrease in agranulocytes.

The use of the antagonist in lot IVB resulted in a definite hypochromic anemia. It is difficult to state definitely whether a folic acid deficiency results in a hypochromic or hyperchronic anemia since no hypochromic anemia developed when sulfasuxidine alone was added to the basal ration. This statement also needs to be qualified by stating that the color index used is based on 12.5 gm of hemoglobin and 7,000,000 RBC being regarded as near normal. If other values for "normal" were used, then the color index figures might be different and different conclusions might be drawn. On the other hand, if the color index in lot VI is taken as near normal, then a slight hypochromic anemia may have developed due to a folic acid deficiency. Anisocytosis was observed in all lots of pigs.

SUMMARY AND CONCLUSIONS

The basal ration, fed for 21 weeks, was inadequate for hematopoiesis in the pig. It is recommended that folic acid be added to the basal ration (containing 6 B-complex vitamins — thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline) when it is used in studying the effects of other vitamins in the pig.

The addition of 10% dehydrated alfalfa meal to the basal ration, for a 13-week period, was of no benefit on growth or efficiency of feed utilization. However, it was beneficial to about the same extent as folic acid, in stimulating hemoglobin and red blood cell formation.

The addition of folic acid or the anti-pernicious anemia liver extract to the basal ration + sulfasuxidine resulted in an increased need for biotin since biotin deficiency symptoms showed up 2 weeks earlier and developed very much more severely than when only sulfasuxidine was added to the basal ration. This may have been caused by some vitamin imbalance. This might suggest the possibility of biotin therapy being needed for human patients being treated with folic acid or the anti-pernicious anemia liver extract. Sulfasuxidine feeding for 21 weeks resulted in the development of only slight symptoms of biotin deficiency.

A normocytic anemia was produced in the pig by adding sulfasuxidine for 21 weeks, to the basal ration. The anemia was prevented by folic acid and to a lesser extent by an anti-pernicious anemia liver extract (at levels both were fed). A more severe anemia was produced by using a crude folic acid antagonist. A combination of biotin and folic acid was more effective than folic acid alone in counteracting the effect of the crude folic acid antagonist.

The effect of sulfasuxidine, over a long period of time, in decreasing gains and efficiency of feed utilization was counteracted almost entirely by a combination of folic acid and biotin. However, sulfasuxidine did not affect gains or efficiency of feed utilization during the first 6 weeks the pigs were on experiment; neither did it affect hemoglobin or erythrocyte formation during the first 13 weeks on experiment. This indicates that sulfasuxidine can be used therapeutically, at levels fed in this experiment, for a considerable length of time without any visible injurious effect on the pig.

The anti-pernicious anemia liver extract had no effect (at level fed) in stimulating hemoglobin or erythrocyte formation when the crude folic acid antagonist was added to the basal ration + sulfasuxidine.

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THE EFFECT OF ENZYMATIC HYDROLYSIS ON THE NUTRITIVE VALUE OF CASEIN

I. DIGESTION OF CASEIN WITH PANCREATIC ENZYMES

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ONE FIGURE

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Processing does at times have a marked effect on the nutritive value of dietary proteins. Heating certain proteins may reduce the nutritive quality presumably due to the formation of new linkages resistant to enzymatic digestion (Block et al., '46; Eldred and Rodney, '46), whereas, moderate heating of other proteins like soybean may increase the nutritive value presumably through facilitating subsequent liberation of methionine during the digestive process (Melnick, Oser and Weiss, '46).

The latter observation, then, raises the question whether or not preliminary enzymatic digestion, before feeding, will alter the rate of absorption of certain of the contained amino acids and thereby change the nutritive value. The data available favor the view that the hydrolysis of a protein reduces, rather than enhances, the nutritive value by destroying some essential constituents. Miller, Robscheit-Robbins and Whipple ('47), for example, suggest that some unidentified substances present in the dietary proteins and absent in mixtures of amino acids are responsible for maintenance of weight in depleted dogs. These unidentified factors may be similar to those reported by Womack and Rose ('46) who have pre-

sented evidence that protein may contain substances which are required for maximum increases in weight, substances not found in amino acid mixtures or certain protein digests. The recent work of Woolley ('41, '46) demonstrates that one or more essential peptides may be furnished by the intact protein and not by a complete hydrolysate, and the absence of these may lower the nutritive value of the hydrolysate.

It was for these reasons that the relationship between the degree of enzymatic hydrolysis and the nutritive value of a protein was studied. The experiment reported here was undertaken to determine the effect of enzymatic predigestion of casein on the nutritive value of the protein. Casein was selected for this study because (a) of the ease of its digestion by pancreatic enzymes and (b) of its high nutritive properties which have been carefully characterized. Digestive enzymes were used to catalyze hydrolysis simulating the type of digestion which takes place in the intestinal tract. The amount of enzyme used was small enough so that supplementation of casein with enzyme protein was not an important factor. Hydrolysates in which 18, 38 and 59% of the amino acids were liberated, were prepared. The nutritive values of casein and these 3 hydrolysates were then determined by measuring their nitrogen balance indexes in adult dogs and their protein efficiencies in rats.

THE ENZYMATIC STUDIES

In order to minimize a supplementation effect due to the addition of enzyme amino acids, the enzymes of crude extracts of beef or hog pancreas were fractionated and the enzymes activated. The procedure utilized is given below.

Purification and activation of tryptic enzymes

One hundred and fifty grams of freshly ground beef or hog pancreas were extracted in a Waring Blendor for 5 minutes with 290 ml of water and 12.5 ml of 6 N H_2SO_4. The pH of the suspension was raised to 4.2 with 10 ml of 6 N NaOH, and the

mixture was filtered. In order to activate the enzyme the pH of the filtrate was raised to about 7.8 and kept at 0°C. (ice water mixture) for approximately 72 hours. This affected an increase in enzyme activity from 17 [1] to approximately 770 units per milliliter and 550 units per milligram of nitrogen. The time necessary for activation can be reduced if a sample of previously activated enzyme solution is added in an amount equivalent to 5% of the total enzyme activity. A further purification is possible through precipitation of enzyme at 0°C. with ethyl alcohol (70%). The unit activity of such alcohol-precipitated enzyme is about 1700 units per milligram nitrogen instead of 550. Crystalline trypsin and crystalline chymotrypsin contain 800 and 1700 units per milligram nitrogen, respectively, according to the test method utilized.

Preparation of carboxypeptidase

Crystalline carboxypeptidases were prepared according to the procedure described by Anson ('37).

Preparation of casein hydrolysate solutions

(1) 18% Hydrolysis. The procedure for digestion is briefly as follows: Eight hundred grams of crude edible casein were suspended in 8 l of distilled water and enough sodium hydroxide (approximately 104 ml of 4 N NaOH) added to bring the pH of the solution to about 7.6. Five grams of the purified tryptic enzymes were added to the solution and the digest kept at 55°C. Aliquots were taken periodically to follow the course of digestion, using the increase of amino nitrogen by formol titration as a criterion. It was found that the digestion reached a maximum of 18% in less than 24 hours. Further addition of purified enzyme at this point did not materially

[1] One unit of tryptic activity is defined as the mount of enzyme which will digest 1 ml of 0.25% casein solution at pH 7.6 and 37.5°C. to the extent of 50% in 15 minutes. The details of the method will be published in the J. Gen. Physiology, 1948 (B. F. Chow and M. W. Peticolas).

increase digestion. After maximum digestion, the whole suspension was lyophilyzed without filtering in order to include in the final preparation all essential amino acids and/or accessory growth factor. The solid material (about 900 gm) was used for biological and chemical analysis. The material contained 13.2% total nitrogen, of which 18% was amino nitrogen by the ninhydrin method.

(2) 40% Hydrolysis. In order to obtain a 40% hydrolysis, a highly purified carboxypeptidase was added to the 18% casein hydrolysate as prepared under (1). Approximately 3 gm of purified carboxypeptidase were added to each 800 gm of the 18% hydrolysate. The degree of digestion is also followed by the formol titration, and after maximal digestion the suspension was lyophilyzed. The solid material was found to contain 13.5% nitrogen, 40% of which was alpha amino nitrogen by the ninhydrin method.

(3) 60% Hydrolysis. A solution of 800 gm of crude casein was made in the usual manner. Four grams of purified hog pancreatic extract which contained, besides the tryptic enzymes and carboxypeptidases, amino polypeptidase were added and the digestion was allowed to proceed at 55°C. for 48 hours; the mixture was then lyophilyzed. The dry powder was found to contain 13.5% total nitrogen, of which 60% was alpha amino nitrogen by the ninhydrin method.

THE BIOLIOGICAL ASSAYS

Protein efficiencies in rats were determined by the Osborne-Mendel method ('26). Fifteen rats were used for each test hydrolysate at the following nitrogen levels in the diet: 1.1%, 1.4% and 1.7%. The nitrogen balance indexes in dogs (K) were calculated according to the following equation:

$$NB = K(AN) + NE_o$$

where NB is nitrogen balance, AN is absorbed nitrogen and NE_o is the excretion of nitrogen on a protein-free diet (Allison, Anderson and Seeley, '46). Seven dogs were fed a protein-free diet for 8 days. The urine and feces samples were

collected the last 3 days to determine NE_o. Each dog was then fed the casein sample at a level of approximately 0.15 gm nitrogen per kilogram body weight per day for 8 days. The first 2 days were an adjustment period after which two 3-day urine samples were collected. Each dog returned to the protein-free diet for 5 days with a 2-day adjustment period and a 3-day urine and feces collection period. Thus, duplicate measurements were made on each dog with an NE_o associated with each sample. These same dogs were then returned to the stock laboratory diet for 2 to 3 weeks and used for another test.

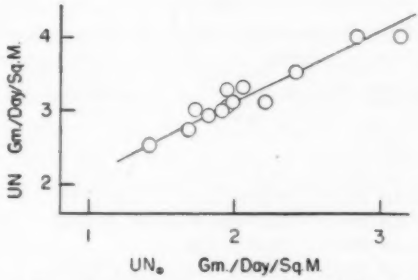

Fig. 1 Urinary nitrogen excretion in dogs fed casein nitrogen against excretion of nitrogen on a protein-free diet.

Previous studies have demonstrated that if absorbed nitrogen (AN) from any one protein is constant, the following relationship should hold:

$$UN = UNo - C$$

where UN is the urinary nitrogen excretion during nitrogen feeding and UNo is the urinary nitrogen excretion while the dogs are receiving a protein-free diet (Allison, Seeley and Ferguson, '47). The value of C is a constant equal to (1—K) (AN) where K is the nitrogen balance index. On the other hand, if there has been some shift in the physiological state of the dog, the values for K will no longer be constant. Data obtained while feeding casein to the dogs in the studies reported here are illustrated in figure 1. The relationship

between UN and UNo is linear with a slope of unity. The constant C is equal to 1.1, and AN is 3.45 gm/day/sq. M. The nitrogen balance index K is equal therefore to 0.68.

Average values for nitrogen balance indexes for casein and for the hydrolysates fed to dogs are recorded in table 1, together with the protein efficiency measurements in the rats. The data demonstrate that predigestion of casein with the enzymes does not alter the utilization of the nitrogen as measured by balance indexes and protein efficiencies. These results can be interpreted to mean that hydrolysis did not

TABLE 1

The effect of degree of hydrolysis on the nutritive properties of casein

DEGREE OF HYDROLYSIS	DIGESTIBILITY IN DOGS	NITROGEN BALANCE INDEX IN DOGS	PROTEIN EFFICIENCY IN RATS	STREPOGENIN CONTENT	
				Actual	Potential
%	%			*Units/gram*	
0	98 ± 0.8 (7)[1]	0.68 ± 0.05	2.3 (15)[1]	0	5
18	98 ± 1.0 (7)[1]	0.67 ± 0.04	2.2 (15)[1]	1	5.5
38	98 ± 1.0 (7)[1]	0.67 ± 0.05	..	5	5.5
53	97 ± 1.8 (7)[1]	0.69 ± 0.08	..	5	5
59	95 ± 1.7 (6)[1]	0.68 ± 0.06	2.4 (14)[1]	5	5

[1] The number inside the parentheses indicates the number of animals used for each determination.

change the nutritive value of the protein. It should be emphasized that ingested casein is rapidly digested by enzymes of the gastro-intestinal tract so that no matter what the preliminary hydrolysis may have been, the liberation of essential amino acids in the gut may be from whole protein and from an hydrolysate quite similar.

DISCUSSION

These observations do not rule out the possibility that complete hydrolysis of casein would alter the retention of nitrogen in the animal by changing the pattern of amino acid absorbed. It is commonly reported and assumed that complete hydrolysis reduces the biological value of a nitrogen source (Supplee and

Clark, '46). These reports are based, however, on feeding hydrolysates made with acid or by feeding mixtures of amino acids. Acid hydrolysis destroys tryptophane and possibly other amino acids which must be restored, and restorations may not always be adequate. Furthermore, the lower biological values observed during the feeding of amino acid mixtures may be associated with the presence of unnatural isomers (Albanese and Irby, '43; Murlin et al., '46). Cannon et al. ('46), on the other hand, found that a mixture of 16 amino acids patterned after casein produced gains in protein-depleted rats equivalent to that yielded by casein itself. Under the conditions of the experiments reported here, however, the hydrolysates reached the gut with certain polypeptides intact, making possible their absorption or their hydrolysis by peptidases in the normal manner. The potential strepogenin content of the casein, for example, was not altered by the enzymatic hydrolyses (see table 1). Thus it would appear that enzymatic hydrolysates, at least of casein, prepared according to methods described in this paper, retain the nutritive properties of the parent protein.

SUMMARY

Casein was hydrolyzed to different degrees (18%, 40%, 60%) through the selective use of pancreatic enzymes under conditions which minimize any destruction of amino acid patterns. It was found that the nitrogen balance indexes and the protein efficiency values of the hydrolysates remained essentially identical with those of the undigested protein.

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THE EFFECT OF ENZYMATIC HYDROLYSIS ON THE NUTRITIVE VALUE OF CASEIN

II. DIGESTION OF CASEIN WITH BACTERIAL AND FUNGAL ENZYMES

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ONE FIGURE

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Woolley ('41) reported that strepogenin, a growth nutrient present in casein, is liberated by the digestion with trypsin but not with pepsin, although the latter carries the hydrolysis to a greater extent. These results led to the suggestion that the position of cleavage of the protein molecule may be a more important factor in determining the nutritive value of a hydrolysate than the degree of hydrolysis. The stepwise hydrolysis of casein (Chow, Allison and White, '48) by purified pancreatic enzymes did not alter the nitrogen balance indexes or the protein efficiencies of hydrolysates as compared to the undigested protein. However, hydrolysis at different positions would yield peptides with different amino acid patterns. This can be done by using enzymes which are not present in the digestive tracts of mammalian experimental animals. This plan was utilized in order to determine whether such predigestion would affect the nutritive value of the resulting hydrolysate.

[1] The author acknowledges the technical assistance of Claire Federbush and Harry Slocum in the nitrogen balance determinations.

EXPERIMENTAL[2]

(1) Enzymes used

Two commercially available enzymes (Rhozyme P-11[3] isolated from fungus and Protease 15[4] isolated from bacteria) were chosen for this study because they were found to split the protein chain of the casein molecule at different positions.

(2) Digestion of casein

The procedure for digestion was as follows: Eight hundred grams crude edible casein[5] were suspended in 8 l of distilled water and enough sodium hydroxide (approximately 104 ml of 4 N NaOH) added with efficient stirring to bring the pH of the solution to about 7.6. Ten grams of the enzyme powder, either Protease 15 or Rhozyme P-11, containing approximately 6% nitrogen were then added to the solution incubated at 37°C. An additional portion of the enzyme (2.5 gm) was added on the third day and the digestion was allowed to continue for 4 additional days. Aliquots were taken periodically to follow the course of the digestion with the formol titration method. After the maximal digestion, the whole suspension was lyophilyzed, in order to minimize the loss of essential amino acids or growth factors that might be contained in an insoluble residue. The yield of the hydrolysate was 850 gm.

(3) Determination of nitrogen balance index

The procedure for the determination of nitrogen balance index is essentially that of Allison, Anderson and Seeley ('46) and has been described in detail in the preceding paper.

[2] The author acknowledges the technical assistance of Lois Barrows for the preparations of hydrolysates.

[3] Rhozyme P-11 obtainable from Rohm and Haas Company, Philadelphia, Pa.

[4] Protease 15 obtainable from Rohm and Haas Company, Philadelphia, Pa.

[5] The Borden Company, New York City, N. Y.

RESULTS

(1) Hydrolysis of casein by different enzymes

If 2 enzymes have sufficient specificity to attack a protein molecule at different points, the addition of the second enzyme to the protein solution previously digested to completion by the first enzyme, should cause a demonstrable further

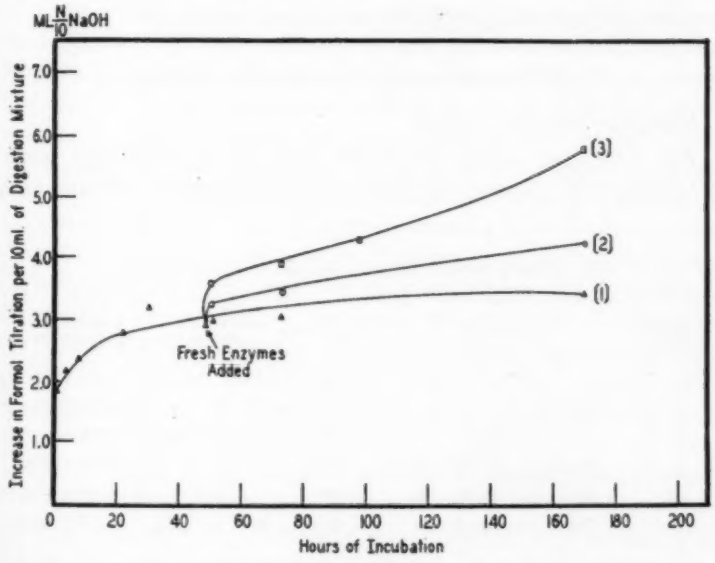

Fig. 1 The effect of addition of Rhozyme P-11 (curve 3) and of Protease 15 (curve 2) on the digestion of a casein previously hydrolyzed by purified pancreatic enzymes (curve 1).

digestion. The same situation should obtain if one reverses the order of addition of enzyme. This increase can be most conveniently followed by the formol titration method which was used to demonstrate that the 2 proteolytic enzymes isolated from bacterial or fungal origins split casein at different parts of the molecule. An example of the experimental data which demonstrate this difference is summarized in figure 1.

It can be noted in figure 1 that the addition of Protease 15 or Rhozyme P-11 to casein previously digested with purified pancreatic enzymes to the maximum extent of 18% (Chow, Allison and White, '48), causes a further increase in alpha amino acid nitrogen. Similar curves were obtained upon the addition either of purified pancreatic enzymes or of Rhozyme P-11 to a casein solution previously digested by Protease 15, or upon the addition of either Protease 15 or purified pancreatic enzymes to the Rhozyme digests of casein.

(2) Determination of nutritive values

Nitrogen balance index:

The nutritive values of casein and its hydrolysates were determined by measuring their nitrogen balance indexes and

TABLE 1

The nitrogen balance index of casein and its hydrolysate

EXPERIMENT NUMBER	SUBSTRATE	DIGESTION	NUMBER OF ANIMALS	AVERAGE K ± STANDARD ERROR
		%		
1	Casein	0.0	9	0.77 ± 0.03
2	Protease digest	18	9	0.73 ± 0.02
3	Rhozyme digest [1] (1)	40	7	0.77 ± 0.03
4	Rhozyme digest (2)	40	4	0.79 ± 0.05
5	Rhozyme digest (3)	40	5	0.72 ± 0.03

[1] Rhozyme digests (1), (2) and (3) were made on 3 separate batches.

their protein efficiencies. The former measures the amount of a protein or a hydrolysate required for the maintenance of nitrogen equilibrium in adult dogs. The latter measures the amount of a protein or of a hydrolysate required for the normal growth of weaned rats.

The results of the nitrogen balance studies (see table 1) indicate that this sample of casein and its 2 hydrolysates have the nitrogen balance indexes of 0.77 (casein), 0.72 to 0.79 (Rhozyme digest) and 0.73 (Protease digest), respectively.

These slight differences are found to be statistically insignificant (Fisher and Yates, '43).[6]

For the determination of protein efficiency, 3 groups of ten 23-day-old rats were fed casein or one of the hydrolysates as the sole source of amino acid nitrogen at the following nitrogen levels in the diet; 1.1%, 1.4% and 1.7%. The weights of individual animals as well as the food consumption were recorded twice a week. The maximum ratios of weight gain to food consumed, i.e., protein efficiency, after 3 weeks of feeding at 1.4% nitrogen level are 2.2 for casein, 2.0 for Rhozyme digest and 2.0 for Protease digest. Therefore, there appears to be no significant difference in the growth promoting properties of the protein or the hydrolysates.

The failure to demonstrate any difference in nutritive qualities measured either in terms of nitrogen balance indexes or in protein efficiency may be taken to mean a lack of appreciable destruction or removal of any limiting essential amino acids or polypeptides necessary for maintenance or for growth requirements either during the digestion or during the lyophilization of the hydrolysates. It also indicates that the amount of nitrogen in the added enzyme was of such a small order of magnitude as not to materially change the amino acid patterns of the hydrolysates.

The presence of an adequate amount of strepogenin or some yet unidentified factor has been shown to be necessary for optimal rat growth (Woolley, '41; Womack and Rose, '46). There were only 2 units of strepogenin [7] per gram of the Protease digest, 5 units per gram of the Rhozyme digest and none in casein itself. The digestion of casein or Protease di-

[6] It should be pointed out that there is a slight but significant difference in the nitrogen balance indexes, reported in this and the preceding paper. They were determined on the same sample of casein, but with 2 sets of dogs kept in 2 separate laboratories and on different preparatory diets before determination. Whether this difference in the index values can be explained on the animal variations alone, is still not certain at this time. This, however, does not invalidate our conclusions since the comparisons of the protein and its respective hydrolysates were performed on the same set of animals.

[7] The author is indebted to Dr. A. Black, of E. R. Squibb & Sons, for the strepogenin determinations.

gest with trypsin liberated 5 units and 3 additional units, respectively, but the strepogenin content of the Rhozyme digest was not influenced by the same treatment.

These experiments provide confirmatory evidence that the 3 enzymes attack different parts of the casein molecule. They demonstrate that neither the position, the extent of cleavage of the casein molecule, nor the type of enzymes used has influenced the nutritive properties of the hydrolysate, even though in one case the content of the growth factors like strepogenin has been liberated but not destroyed.

SUMMARY

Casein was digested by Protease 15 and Rhozyme P-11 under conditions which minimized any change from the original amino acid composition of casein either by the removal or destruction of amino acids or by the addition of any significant amounts of amino acids from the enzymes. When fed to rats and dogs, the 2 hydrolysates prepared by the digestion of casein in neutral medium were found to yield the same nitrogen balance indexes and protein efficiencies as the whole protein.

These results suggest that neither the degree of digestion up to 40% nor the mode of attack necessarily affect the nutritive values of casein hydrolysates, so long as essential amino acids and perhaps the growth factors in casein are not destroyed.

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STUDIES IN AMINO ACID UTILIZATION

III. THE ROLE OF THE INDISPENSABLE AMINO ACIDS IN MAINTENANCE OF THE ADULT ALBINO RAT [1]

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Department of Pathology, University of Chicago, Illinois

ONE FIGURE

(Received for publication March 25, 1948)

INTRODUCTION

Because of conflicting evidence reported by 2 groups of workers, uncertainty exists concerning the role of some of the "essential" amino acids for maintenance of the adult white rat. Thus, 1 group (Wolf, '38; Wolf and Corley, '39) has reported that, except for arginine, all the amino acids essential for growth in young rats are essential, also, for maintenance of nitrogen equilibrium in adult rats. Another group using similar animals, rations and techniques (Burroughs, Burroughs and Mitchell, '40) has concluded that only 5 of the 10 amino acids essential for growth, viz., tryptophane, threonine, isoleucine, valine and methionine, are essential for maintenance of nitrogen balance in the adult rat.

[1] The research which this paper reports was undertaken in cooperation with the Navy Department Office of Naval Research and the Committee on Food Research of the Quartermaster Food and Container Institute for the armed forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The work has been aided, also, by The National Livestock and Meat Board, The Douglas Smith Foundation for Medical Research of The University of Chicago, and the Allen B. Wrisley Company.

Other workers, however, have found that 8 or 9 of the amino acids essential for growth of the rat are also necessary for the maintenance of nitrogen equilibrium in the dog (Rose and Rice, '39) and man (Rose et al., '43). In view of these findings one would not expect that 3 of the amino acids essential both for growth in the rat and for maintenance of nitrogen balance in man or dog, viz., lysine, leucine and phenylalanine, should be nonessential for maintenance of nitrogen balance in the adult rat. More recently, Neuberger and Webster ('45) have shown that at least 1 of these, lysine, is essential for the maintenance of body weight as well as liver protein and concentration of blood protein in the adult rat. Mitchell ('47) has criticized this work because ad libitum feeding was used and has suggested that the results may indicate inadequate caloric intake rather than the indispensability of lysine. He has offered an hypothesis to explain the apparent species difference in the essential amino acid requirements for maintenance between man (or the dog) and the rat. Because the rat grows at a much faster rate than man or the dog, he suggests that this species might retain in maturity a greater capacity to synthesize certain of the essential amino acids. He believes that this synthesis might be adequate to meet the animal's maintenance needs.

We undertook this study because of earlier findings in the adult protein-depleted rat that, with the exception of arginine, all the essential amino acids were necessary for effective protein-repletion when fed in a ration adequate in other dietary constituents and containing a mixture of purified amino acids as the sole source of dietary nitrogen (Frazier et al., '47; Benditt et al., '47). For example, whenever any one of the 9 essential amino acids was omitted from the ration the animal promptly lost appetite and weight and failed to regenerate serum protein or hemoglobin. This trend was quickly reversed, however, when the missing amino acid was added to the ration. In the following study the response of the normal adult rat to certain amino acid deficiencies was investigated in a similar manner. In addition, the nitrogen

balance technique and force-feeding method were used in some experiments. The results indicate that for maintenance of the adult male rat all the amino acids now known to be essential for growth are required, with the possible exception of arginine.

EXPERIMENTAL METHODS

The rations used were patterned after those employed in former experiments in this laboratory with protein-depleted rats (Frazier et al., '47). These will be referred to hereafter as rations A, B, and C. Ration A contained a mixture of 16 purified amino acids; ration B, the 10 essential amino acids; and ration C, the essential amino acids minus arginine (table 1). For further details concerning composition of the diets, sources and purity of the amino acids, and the method of omitting an amino acid from the ration, the reader is referred to Frazier et al. ('47). The quantities of each amino acid contained in the daily allotment of the basal diets employed by Wolf and Corley ('39) and Burroughs, Burroughs and Mitchell ('40) are also recorded in table 1 for purposes of comparison. It should be noted that the proportions of the various amino acids in our rations were modeled after those in casein whereas the previous workers used mixtures which in most instances contained an amount of each amino acid equivalent to 4 mg of nitrogen daily.

Sixty-three male albino rats, of the Sprague-Dawley strain, weighing from 262 to 325 gm, and approximately 5 months of age were used. Prior to the beginning of the experiment the rats had been fed a ration containing 22% casein (3C), for approximately 3 months (Wissler et al., '45). Before each experiment was started initial weights were obtained after a 12-hour fast. In the experiments in which rations lacking an essential amino acid were tested, pairs of rats of comparable weights were used, 1 being fed ration A and its partner this ration devoid of the amino acid in question. In most instances both rations were fed for 20 days, during which time

TABLE 1

Amino acid mixtures employed in maintenance experiments

AMINO ACIDS	MG OF AMINO ACID PER RAT PER DAY AS ADDED TO RATION								MG PER RAT PER DAY OF UTILIZABLE ESSENTIAL AMINO ACIDS [1]				
	Form	Diet A	Diet B	Diet C	Form	Wolf and Corley Ration no. 43	Form	Burroughs et al. Basal ration	Diet A [2]	Diet B [2]	Diet C [2]	Wolf and Corley Ration no. 43	Burroughs et al. Basal ration
Arginine	HCl, L	76.7	119.9	....	..	....	HCl, L	15.0	63.4	99.1	....	...	12.4
Histidine	HCl, H_2O, L	52.0	81.6	87.4	HCl, H_2O, L	20.0	HCl, H_2O, DL	20.0	38.5	60.4	64.7	14.8	14.8
Isoleucine	DL	200.0	314.4	336.6	L	37.4	DL	37.4	100.0	157.2	168.3	37.4	18.7
Leucine	L	190.0	294.3	313.1	L	37.4	DL	37.4	190.0	294.3	313.1	37.4	18.7
Lysine	HCl, H_2O, L	145.5	229.6	245.1	2HCl L	31.4	HCl DL	26.1	106.0	167.2	178.5	20.9	13.0
Methionine	DL	54.0	84.6	90.6	DL	42.6	DL	42.6	54.0	84.6	90.6	42.6	42.6
Phenylalanine	DL	80.0	125.7	134.6	L	47.2	DL	47.2	80.0	125.7	134.6	47.2	47.2
Threonine	DL	120.0	188.6	201.8	Synthetic	113.4	DL	34.0	60.0	94.3	100.9	28.4	17.0
Tryptophane	DL	27.7	43.5	46.6	L	24.3	L	29.2	27.7	43.5	46.6	24.3	29.2
Valine	DL	216.0	338.4	362.4	L	33.4	DL	33.4	108.0	169.2	181.2	33.4	16.7
Alanine	DL	86.5	....	....	..	....	DL	25.4	....	....	....	...	...
Aspartic acid	DL	97.0	....	....	..	....	DL	38.0	....	....	....	...	...
Cystine	L	5.6	....	....	..	....	L	34.3	....	....	....	...	...
Glutamic acid	L	366.5	....	....	..	....	DL H_2O	47.2	....	....	....	...	...
Glycine	..	7.7	....	....	..	....	..	21.4	....	....	....	...	...
Tyrosine	L	99.0	....	....	..	....	L	51.7	....	....	....	...	...
Serine	..	....	....	....	..	....	DL	30.0	....	....	....	...	...
Norleucine	..	....	....	....	..	....	DL	37.4	....	....	....	...	...
Proline	..	....	....	....	..	....	L	32.9	....	....	....	...	...
Hydroxyproline	..	....	....	....	..	....	L	37.4	....	....	....	...	...

[1] For purposes of calculation of utilizable essential amino acids the D forms of tryptophane, phenylalanine, methionine and histidine have been assumed to be utilizable.

[2] The amino acids listed for rations A, B and C represent the quantities in 15 gm of each ration (with the exception of the high fat ration where they represent the quantities in 9 gm of ration). These figures used with the diet consumption data listed in tables 2 to 5 make it possible to calculate individual amino acid intakes for any period.

the daily food consumption was recorded and the rats were weighed at fixed intervals. In some experiments the rations were interchanged at the end of the 20-day period so that the rat which had received the deficient ration was offered the complete one, and vice versa, for an additional 10 days. When nitrogen balances were determined, however, it was always during the first 14 days of the feeding period.

Nitrogen balance determinations

Two consecutive 7-day nitrogen balances measured for each rat receiving an amino acid ration served as mutual checks. In each instance these were preceded by a preliminary collection period of 7 days during which the animal was fed a casein ration which furnished 216 mg of nitrogen daily (Frazier et al., '47). Urine for each period was collected on filter paper impregnated with boric acid. Feces and waste food (the latter usually minimal) were collected separately each day. The boric acid papers were rinsed free of their nitrogen with 1% sulfuric acid. Duplicate samples of urine and feces were then analyzed for nitrogen by the Kjeldahl method. The net nitrogen intake for each period was calculated from the nitrogen content of the diet, multiplied by the amount consumed in 7 days from which the waste food had been subtracted. The nitrogen output for the week was the sum of the urinary and fecal nitrogen.

Force-feeding method

In 1 experiment (table 3) force-feeding via stomach tube was employed in order to equalize food intakes. The technique of Shay and Gruenstein ('46) was used and found to be satisfactory. The rats were fed thrice daily at approximately 5-hour intervals, using a special high-fat basal diet. This diet contained in 9 gm the same quantities of all constituents as were contained in 15 gm of ration A, but was concentrated by the substitution of fat for a caloricallv equivalent quantity of carbohydrate. In order to avoid any disturbance which

[illegible] The amino acids listed for rations A, B and C represent the figures used with the diet consumption data listed in tables [illegible] where they represent the quantities in 9 gm of ration). These figures [illegible] possible to calculate individual amino acid intakes for any period.

might result from overloading the gastrointestinal tract, the rats were given only 1.5 gm of ration per feeding the first day, 2.0 gm the second day and 3.0 gm the third day and thereafter. The ration was carefully weighed before each feeding, diluted with a little water and mixed in a small mortar, and then transferred quantitatively to a 10-ml syringe. After each feeding the syringe and stomach tube were rinsed with a small quantity of dilute sulfuric acid. At the end of the nitrogen balance period this pooled rinse water was analyzed for nitrogen and the amount subtracted from the dietary intake for the period.

EXPERIMENTAL FINDINGS

The role of the "nonessential" amino acids in maintenance was first determined in 6 rats (nos. 1–6 table 2), each rat being fed over the 20-day period the ration containing only the 10 essential amino acids (ration B) (Rose, '38). The results show that in every instance food consumption was good, weight was maintained, and nitrogen balance, when measured (nos. 3, 4, 5 and 6), was strongly positive. Therefore, the "nonessential" amino acids may be considered as dispensable, also, for maintenance of appetite, weight and nitrogen balance in the adult rat.

Six additional rats (nos. 7–12) were then fed ration C. This ration, containing only the 9 essential amino acids, appeared in some instances to depress appetite. In 3 of the 6 rats, weight losses of from 5 to 20 gm occurred in the 20-day period (table 2). However, food consumption with rats 11 and 12 was satisfactory and these animals maintained strongly positive nitrogen balances. In 3 of the 6 rats, moreover, weight was maintained or gains were recorded. Evidently, therefore, the absence of arginine exerts a somewhat variable effect upon weight and appetite. Nevertheless, the results obtained with rats 11 and 12 indicate that arginine is not necessary for strongly positive nitrogen balance and maintenance of weight when food consumption is satisfactory. Since it previously was shown that a lack of arginine did not interfere with weight

TABLE 2

Weight changes and nitrogen balances of adult well-nourished rats fed amino acid mixtures

ANIMAL NO.	RATION [1]	20-DAY PERIOD		NITROGEN BALANCE							
				First 7 days				Second 7 days			
		Wt. gain or loss	Ration eaten	N intake	Fecal N	Urinary N	N Balance	N intake	Fecal N	Urinary N	N Balance
		gm	gm	mg	mg	mg	mg	mg	mg	mg	mg
1	B	+ 8	294	....	....	....	....	....	....	....	....
2	B	± 0	298	....	....	....	....	....	....	....	....
3	B	+18	294	1617	159	1308	+150	1612	159	1278	+175
4	B	+11	295	1626	216	1228	+182	1628	180	1209	+239
5	B	+12	300	1645	236	1275	+134	1644	206	1304	+134
6	B	+23	320	1637	260	1114	+263	1637	270	1324	+ 43
7	C	—12	257	....	....	....	....	....	....	....	....
8	C	—20	239	....	....	....	....	....	....	....	....
9	C	— 5	288	....	....	....	....	....	....	....	....
10	C	— 1	286	....	....	....	....	....	....	....	....
11	C	+26	269	1352	165	834	+353	1253	212	716	+325
12	C	+15	282	1348	178	861	+309	1372	212	975	+185
13	A [2]	+14	294	1564	135	1061	+368	1617	133	1167	+317
14	—Tryp.	—54	280	1478	235	1472	—229	1537	260	1549	—274
15	A [3]	+ 6	298	1617	181	1113	+313	1617	159	1175	+283
16	—Meth.	—40	283	1534	214	1550	—230	1550	209	1530	—224
17	A [4]	+13	295	1474	208	1170	+ 96	1500	178	1153	+169
18	—Iso.	—84	138	1068	101	1155	—188	804	154	1132	—717
19	A [5]	+ 9	296	1465	180	1097	+188	1484	133	1124	+227
20	—Val.	—67	182	1006	121	1098	—212	1055	228	1240	—412
21	A [6]	+ 5	296	1478	208	1078	+192	1485	193	1207	+ 85
22	—Threo.	—42	288	1460	235	1619	—394	1473	220	1692	—439

[1] The minus sign preceding the abbreviation of an amino acid indicates that this amino acid was omitted from ration A during this period.

[2] Identical with ration A except that it contained 13 mg of available tryptophane per daily portion.

[3] Identical with ration A except that it contained 35 mg of available methionine per daily portion.

[4] Identical with ration A except that it contained 65 mg of available isoleucine per daily portion.

[5] Identical with ration A except that it contained 60 mg of available valine per daily portion.

[6] Identical with ration A except that it contained 25 mg of available threonine per daily portion.

gain in the protein-depleted adult rat (Frazier et al., '47) where presumably the needs of tissue synthesis and maintenance must both be met, it would follow that it is not necessary for maintenance of the adult rat. The low urinary nitrogen output of the animals receiving ration C should receive further investigation.

We then studied the influence of a lack of tryptophane, valine, methionine, isoleucine and threonine, removed singly, upon weight, food consumption and nitrogen balance. Five pairs of normal adult rats were used with a slightly modified ration A serving as a control (see footnotes, tables 2 and 3). As can be seen, in every instance the lack of any one of these amino acids led to a marked loss of weight, and a negative nitrogen balance in each 7-day period. The effect upon food consumption, however, was variable. Thus, the rations lacking tryptophane, methionine and threonine led to less decrease in appetite than did those deficient in valine or, especially, isoleucine. The results leave little doubt, however, about the essentiality of each of these 5 amino acids for maintenance of the normal adult male rat, whether one measures maintenance of weight or of nitrogen balance.

Because lysine, leucine, histidine and phenylalanine were considered by Burroughs, Burroughs and Mitchell ('40) as non-essential for the maintenance of nitrogen balance, the effect of their dietary absence was next determined. The results were as follows: A dietary lack of lysine over the 20-day period led to a marked loss of weight in 4 rats tested (table 3), with a variable reduction in food consumption (from 2 to 26% in comparison with that of ration A). Although nitrogen balance was determined in only 1 rat, this was negative despite an almost complete consumption of the ration. Moreover, when the rations were interchanged for an additional 10-day period, weight losses of 11 and 17 gm were recorded for the 2 rats (23D and 24D) receiving the lysine-deficient diet, although the food consumption of these 2 animals equalled that of the 2 control rats which gained 22 and 14 gm, respectively, on ration A. When either histidine, leucine or phenyl-

TABLE 3

Weight changes and nitrogen balances of adult well-nourished rats fed amino acid mixtures

ANIMAL NO.	20-DAY PERIOD			10-DAY PERIOD			NITROGEN BALANCE							
							First 7 days				Second 7 days			
	Ration [1]	Wt. gain or loss	Ration eaten	Ration [1]	Wt. gain or loss	Ration eaten	N intake	Fecal N	Urinary N	N Balance	N intake	Fecal N	Urinary N	N Balance
		gm	*gm*		*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
23C	A	+11	284	—Lys.	—17	147								
24C	A	+10	285	—Lys.	—11	137								
25C	A	+19	286											
26C	A [2]	+ 1	291				1369	167	924	+278	1360	194	1038	+ 28
23D	—Lys.	—30	223	A	+22	145								
24D	—Lys.	—26	265	A	+14	135								
25D	—Lys.	—26	282											
26D	—Lys.	—23	294				1324	212	1303	—136	1358	215	1190	— 47
27C	A	+11	266	—Hist.	—19	100								
28C	A [3]	+ 9	298				1469				1489	243	1102	+154
27D	—Hist.	—41	173	A	+27	144								
28D	—Hist.	—46	211				1196	134	1075	— 13	1204	220	1128	—144
29C	A	+12	285	—Leuc.	—17	116								
30C	A	+ 6	273	—Leuc.	—26	100								
31C	A	+14	296											
32C	A [4]	— 5	297				1477	194	1073	+210	1481	255	1037	+189
29D	—Leuc.	—43	189	A	+18	143								
30D	—Leuc.	—45	202	A	+22	141								
31D	—Leuc.	—49	228											
32D	—Leuc.	—48	246				1235	204	1067	— 36	1233	167	1215	—144
33C	A	+ 2	288	—PA	—22	116								
34C	A	+ 9	293											
35C	A [5]	+ 8	297				1525	209	1242	+ 63	1538	188	1124	+194
33D	—PA	—53	185	A	+15	149								
34D	—PA	—54	206											
35D	—PA	—28	243				1387	178	1379	—170	1335	202	1247	—114

[1] The minus sign preceding the abbreviation of an amino acid indicates that this amino acid was omitted from ration A during this period.
[2] Identical with ration A except that it contained 20 mg of available lysine per daily portion.
[3] Identical with ration A except that it contained 15 mg of available histidine per daily portion.
[4] Identical with ration A except that it contained 75 mg of available leucine per daily portion.
[5] Identical with ration A except that it contained 30 mg of available phenylalanine per daily portion.

alanine was removed singly from ration A, both food consumption and weight declined; and during this time all animals tested were in negative nitrogen balance. When, however, the rations were interchanged, there was a prompt reversal in the pattern of food consumption and weight (table 3 and fig. 1). Thus, although the absence of lysine affected appetite and weight loss less severely than did the absence of the other 3 essential amino acids, there was little difference in the total negative nitrogen balance for the 14-day period, whether this was due to a deficiency of lysine, histidine or leucine. On the other hand, the absence of phenylalanine resulted in a more severely negative nitrogen balance but with less depression of appetite than did the absence of either histidine or leucine.

It is of interest that although a lack of a single essential amino acid in the ration affected appetite adversely in the well-nourished rat, it did so less severely than in the protein-deficient rat (Frazier et al., '47). Whereas the latter animal's appetite usually deteriorates within a day or two, in the normal well-nourished rat this effect may not manifest itself for 3 or 4 days (fig. 1). With both types of animal, moreover, the least effect upon appetite resulted from a deficiency of lysine.

In the work of Burroughs, Burroughs and Mitchell, it was reported that norleucine could substitute for either leucine or lysine, and tyrosine for phenylalanine. In our experiments this has not been true (table 4). For example, when norleucine was substituted for either leucine or lysine (rats 31S, 36S and 37S) there was a concomitant loss of appetite and weight, the weight loss being the same or greater than that of the animals fed a diet deficient in either lysine or leucine. The substitution of tyrosine for phenylalanine (rat 34S) also failed to prevent a loss of weight, although the weight decrease was somewhat less than it had been with phenylalanine deficiency alone (rat 34D). The latter observation is similar to the findings of others in growing rats (Rose and Womack, '46).

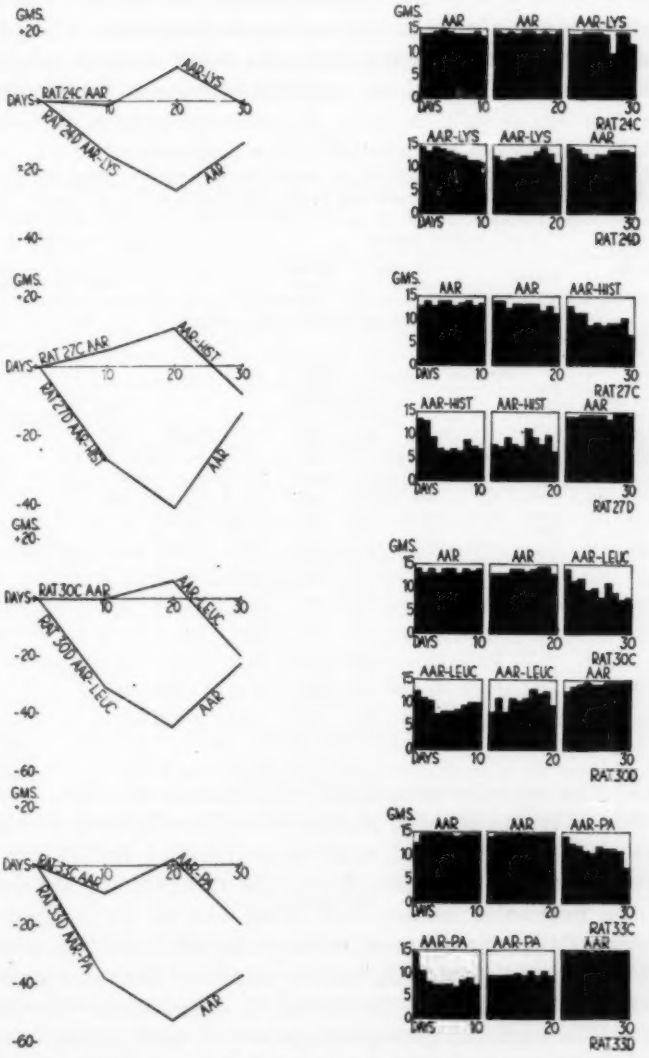

Fig. 1 Comparisons of weight changes and diet consumptions in adult normal rats fed ration A (AAR) and ration A devoid of 1 amino acid.

It is obvious that one cannot evaluate adequately the consequences of an acute amino acid deficiency until it has been shown that a similar effect will also occur with an adequate caloric intake. Therefore, experiments were performed in

TABLE 4

The effect of substituting norluecine for leucine, norleucine for lysine and tyrosine for phenylalanine upon weight and food consumption of normal adult rats

ANIMAL NUMBER	20-DAY PERIOD			10-DAY PERIOD		
	Ration	Wt. gain or loss	Ration eaten	Ration	Wt. gain or loss	Ration eaten
		gm	gm		gm	gm
31S	Norleucine for leucine	—59	207	....	....	...
36C	A	+27	290	Norleucine for lysine	—17	137
37C	A	+27	267	Norleucine for lysine	—14	120
36S	Norleucine for lysine	—27	209	A	+36	143
37S	Norleucine for lysine	—18	233	A	+31	142
34S	Tyrosine for phenylalanine	—43	230	....	....	...

which the animals were force-fed, by stomach tube, rations devoid of lysine, leucine, histidine or phenylalanine (table 5). Pairs of rats were fed equal quantities of ration, one receiving the high fat ration A and the other this ration devoid of the particular amino acid being tested. It is apparent (table 5) that the removal of any one of these amino acids led to a loss of weight and a negative nitrogen balance, despite the comparable intake of all other dietary constituents. The negative nitrogen balances in each animal receiving a deficient mixture were associated with a substantial rise in urinary nitrogen as compared to the preliminary balance

period in which the animals consumed equal quantities of nitrogen from casein. It is of interest that the rat fed the histidine-deficient diet maintained a positive nitrogen balance for the first 7-day period but that during the second period it showed a net negative nitrogen balance. This is the only evidence we have obtained which indicates that histidine in the rat as in man (Rose et al., '43) may temporarily, at least, be dispensable for nitrogen balance.

TABLE 5

Weight changes and nitrogen balance data of adult well-nourished rats force-fed amino acid rations for 14 days

ANIMAL NO.	RATION	14-DAY PERIOD		NITROGEN BALANCE							
				First 7 days				Second 7 days			
		Wt. gain or loss [1]	Ration eaten	N intake	Urinary N	Fecal N	N Balance	N intake	Urinary N	Fecal N	N Balance
		gm	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
38	A	+ 6	101.8	1159	969	103	+ 87	1348	1059	111	+188
39	—Lys.	—11	102.0	1093	1151	110	—168	1277	1276	118	—117
40	A	+ 6	107.3	1171	944	114	+113	1445	1007	106	+332
41	—Leuc.	—30	101.9	1121	1101	120	—100	1306	1449	94	—237
42	A	+15	102.4	1172	780	110	+282	1351	932	87	+332
43	—PA [2]	—10	97.8	1160	1110	110	— 60	1326	1427	117	—218
44	A	+15	102.8	1168	885	124	+159	1352	1004	91	+257
45	—Hist.	—17	102.0	1144	1006	97	+ 41	1329	1343	115	—129

[1] Since all the animals lost weight during the first 2 days of the experiment in which they were being fed small amounts of ration the weight changes are calculated for the final 12 days of the experiment when the caloric intake was adequate.

[2] Phenylalanine.

DISCUSSION

What criterion should be used to measure the qualitative amino acid needs for maintenance of the adult animal? The answer to this question obviously depends upon how one defines "maintenance." In our study we have been primarily concerned with the maintenance of health, as evidenced by the preservation of weight and appetite as well as nitrogen

balance. Therefore, we have utilized a relatively long experimental period of 14 to 20 days; we have employed an amino acid mixture patterned after a natural food protein, casein; and we have offered somewhat larger quantities of amino acids to each rat daily than did either of the 2 groups who have extensively studied this problem previously (Wolf and Corley, '39; Burroughs, Burroughs and Mitchell, '40).

These investigators, on the other hand, were primarily interested in determining whether the endogenous losses of nitrogen were replaced by feeding relatively small quantities of amino nitrogen (table 1) for short periods of time (3–7 days). Their amino acid mixtures resembled no natural food protein but were constructed by the empirical method of including equal quantities (usually 4 mg) of nitrogen from each amino acid. Both of these groups apparently had difficulty in obtaining satisfactory consumption of their "complete" amino acid mixture and had to resort to force-feeding. In our experience the level of the rat's voluntary food consumption is a valuable indication of its state of well being. Whenever its appetite is definitely depressed by the absence or reduction of an essential food constituent it is probable that this dietary deficiency is impairing the animal's health. Even with force-feeding technique, Wolf ('38) states that his animals lost hair and did not thrive and his tabulated data show that steady weight losses often occurred even when the animal was in positive nitrogen balance. Recent evidence concerning the optimal proportions of the various essential amino acids necessary for basic protein needs (Mitchell and Block, '46; Cannon, '47, '48) suggests that an imbalance of their amino acid mixtures may have led to the failure of appetite and health noted by the other workers.

At present we are unable to explain the divergent results obtained by Wolf and Corley ('39) and Burroughs, Burroughs and Mitchell ('40). It should be pointed out, however, that the collection and measurement of small quantities of nitrogen in samples of urine and feces, the necessity for purity of the amino acids and the assurance of their absence

from other dietary constituents make this a very exacting biologic experiment. It is not surprising, therefore, that many of these problems may only yield to study by using varying methods of approach.

Our results indicate that, for the normal adult male rat, 9 of the amino acids essential for growth are essential, also, for the maintenance of appetite, weight and nitrogen balance. This means, we believe, that as in the case of the protein-depleted rat these amino acids cannot be synthesized in sufficient quantities to replace those which are being lost in the processes of catabolism. Due, evidently, to the dynamic state of protein metabolism, this steady daily loss of essential amino acids must be quickly replaced by definite quantities of these amino acids if the animal's tissues and health are to be maintained. The essential amino acids must be regarded, therefore, as dietary expendables to be replaced constantly. When there is an acute deficiency of a single essential amino acid, even in the normal animal, the tissues apparently cannot supply the missing amino acid in time or in sufficient quantity for combination with those ingested in an incomplete amino acid ration. In consequence, the animal loses appetite and weight and goes into negative nitrogen balance. It would appear, therefore, that neither the protein-depleted nor the well-nourished adult rat has available stores of single essential amino acids which can be utilized in times of need. In either instance the symptoms which appear are those of acute amino acid deficiency; and this can be corrected only by replacement of the missing dietary essentials.

In subsequent reports from this laboratory the quantitative requirements of the adult rat for each of these 9 essential amino acids to maintain nitrogen balance, as well as normal weight and appetite, will be contrasted with the requirements of the protein-depleted rat for optimal tissue synthesis.

SUMMARY AND CONCLUSIONS

The qualitative amino acid requirements for maintenance of the normal adult male rat have been measured using the

criteria of preservation of appetite and weight as well as nitrogen balance. A purified amino acid mixture patterned after the composition of casein served as the source of dietary nitrogen in the basal ration. The effects upon appetite and weight following removal of each of the 9 essential amino acids and all of the non-essential amino acids have been observed, as well as the effects of substituting norleucine for leucine or lysine and tyrosine for phenylalanine. When single amino acid deficiencies were studied, pairs of comparable rats, one receiving the complete ration and one the incomplete, were used. In some instances these rations were interchanged at the end of the first 20 days and the experiment was continued for an additional 10 days. Nitrogen balance determinations were made in representative animals during the first 14 days of the experiment, two 7-day collection periods serving as mutual checks. In 1 experiment the force-feeding technique was used to equalize the nitrogen and caloric intakes between the deficient and control rats.

The results may be summarized as follows:

1. The normal adult rat maintains appetite and weight when receiving rations in which only the 10 amino acids essential for growth are present.

2. The removal of arginine from this mixture produces a variable depression of appetite. When appetite is maintained, weight and a positive nitrogen balance are preserved.

3. The absence of each of the remaining 9 essential amino acids from the basal ration, although producing a variable depression in appetite, always leads to weight loss and a negative nitrogen balance. The absence of tryptophane, methionine and threonine does not produce the marked loss of appetite observed with the same deficiencies in the protein-depleted rat. The absence of lysine led to only slight interference with voluntary food consumption in both the protein-depleted and normal adult rat. In most instances the effect on food consumption was not quite so acute or marked as that observed previously in the protein-depleted rat.

4. Substitution of norleucine for leucine or lysine led to decreased appetite and weight loss similar to those produced by a deficiency of these amino acids. Substitution of tyrosine for phenylalanine resulted in a loss of appetite and weight somewhat less severe than that produced by phenylalanine deficiency.

5. Despite equalization of the food intakes of the rats deficient in lysine, leucine, histidine and phenylalanine with their controls by means of the force-feeding technique the animals on deficient rations lost weight and were in negative nitrogen balance.

6. Our results indicate that the same 9 essential amino acids required for growth of the young rat are necessary for maintenance of appetite, weight and nitrogen balance in the adult rat.

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BRAIN EXCITABILITY IN PYRIDOXINE-DEFICIENT RATS[1]

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The spontaneous convulsive seizures of pyridoxine-deficient rats described by Chick, El Sadr and Worden ('40) and Daniel, Kline and Tolle ('42) offer 1 means for the study of the metabolic determinants of brain function. If the specific aberrations induced by pyridoxine deficiency can be correlated with abnormal behavior it can be concluded that processes involving pyridoxine are among the factors responsible for normal brain function. Accordingly, the work described below was done on rats which showed an increased brain excitability as a result of pyridoxine deficiency.

METHODS

Male rats obtained from the Sprague-Dawley Farm were used. A few of the animals were very young, weighing between 40 and 60 gm, but the majority weighed from 125 to 250 gm.

The vitamin B_6-deficient diet had the following percentage composition: sucrose 58, "vitamin-free" casein[2] 27, lard 5, hydrogenated cottonseed oil[3] 5, salt mixture (Hubbell, Mendel and Wakeman, '37) 4, and cod liver oil 1. The following amounts of vitamins (in milligrams) were added to each kilogram: thiamine hydrochloride 20, riboflavin 30, calcium pan-

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[2] Labco.

[3] Crisco.

tothenate 50, nicotinamide 100, inositol 100, p-aminobenzoic acid 600, 2-methyl-naphthoquinone 5, choline hydrochloride 500. In some cases the appearance of deficiency was hastened by the incorporation of 2,4-dimethyl-3-hydroxy-5-hydroxymethyl-pyridoxine (desoxypyridoxine)[4] in the diet. The desoxypyridoxine was added to the extent of 10 or 50 mg to each kg of deficient diet. Identical results were obtained with the 2 concentrations in rats of the same size, but both were more rapidly effective in young than in adult rats. In other experiments, DL-trypophane (10 gm/kg) or L-glutamic acid (20 gm/kg) was added. The control diet consisted of the basic diet with the addition of 50 mg pyridoxine hydrochloride/kg.

When given by subcutaneous injection, pyridoxine hydrochloride was administered as the unneutralized aqueous solution in a dose of 5 mg/rat. Xanthurenic acid was given by subcutaneous injection of a neutralized 0.1% aqueous solution.

Electroshock thresholds were determined as previously described (Davenport and Davenport, '48). The electroshock threshold is the smallest amount of current required to produce a detectable clonic seizure. Under rigidly controlled conditions its value, expressed in milliamperes (mA), is nearly constant from day to day but increases slowly in the normal rat from 10 mA at 50 gm to 20 mA at 250 gm.

Cellular hydration was accomplished by the method of Darrow and Yannet ('35). Isosmolar glucose solution, 10 ml/100 gm body weight, was injected intraperitoneally and was withdrawn 4 hours later, after it had been allowed to equilibrate with the extracellular fluid.

The usual qualitative test for xanthurenic acid in urine, described by Lepkovsky, Roboz and Haagen-Smit ('43), was employed. This consists of the development of a green pigment when a ferric salt is added to the urine and the pH is adjusted approximately to 8.

Blood for chemical analysis was removed from the inferior vena cava under intravenous pentobarbital anaesthesia.

[4] The desoxypyridoxine was generously supplied by Merck and Co.

Plasma was separated using the anaerobic technique of Davenport ('47). The skulls of the exsanguinated animals were opened, the brains removed, and the cerebral cortices separated from the major portion of the white matter. The water content of brain cortex and of plasma was determined by drying samples to constant weight at 105°C. Chloride was determined in plasma by the method of Van Slyke and Sendroy ('23) and in brain by the same method, using the Sunderman and Williams ('33) technique of alkaline digestion. A Perkin-Elmer flame photometer was used in the analysis of dry-ashed brain or plasma for sodium and potassium. The plasma pH was measured with a glass electrode, and the total carbon dioxide content was determined by the method of Van Slyke and Neill ('24).

Blood which was to be analyzed for lactic or pyruvic acid was withdrawn from the inferior vena cava with chilled syringes and measured at once into tubes containing the chilled precipitating reagents. The method of Barker and Summerson ('41) combined with the copper tungstate precipitation method of Somogyi ('31) was used in analyzing for lactic acid. Pyruvic acid was estimated by the method of Long ('42).

RESULTS

Vitamin B_6 deficiency and electroshock threshold

Table 1 is a summary of the effects of injected pyridoxine on the electroshock thresholds of control and vitamin B_6-deficient rats. The 6th and 8th columns show the mean change in electroshock threshold 5 and 24 hours, respectively, after the rats were given subcutaneous injections of pyridoxine hydrochloride. A comparison was made of the threshold of each rat before, 5 hours after, and 24 hours after the injection of pyridoxine, and the significances of the differences, calculated by Fisher's ('36) method, are given in columns 7 and 9.

The pair-fed controls of group 1 showed no change in electroshock threshold after administration of pyridoxine. The rats in group 2, which had no acrodynia and excreted no

xanthurenic acid after 5 to 6 weeks on the deficient diet, likewise showed no change after injection of the vitamin. In the moderately deficient rats of groups 3 and 4 there was a highly significant rise of threshold within 5 hours and a further rise

TABLE 1

Changes in electroshock threshold in rats receiving 5 mg pyridoxine

GROUP		ACRODYNIA	XANTHURENIC ACID	n [1]	CHANGE IN ELECTROSHOCK THRESHOLD			
					5 Hours		24 Hours	
					Change mA	P [2]	Change mA	P [2]
1	Controls	0	0	24	—0.1	0.4	—0.2	0.9
2	Not deficient after 5–6 weeks on deficient diet	0	0	7	—0.1	0.7	+0.1	0.8
3	Deficiency produced by basic deficient diet for 3–6 weeks	++	+++	20	+3.1	<0.01	+3.5	<0.01
4	Desoxypyridoxine for 15 days	++	+++	13	+2.2	<0.01	+3.0	<0.01
5	Desoxypyridoxine for 25 days	++++	+++	7	+1.2	0.2	+2.9	<0.01
6	Desoxypyridoxine for 5 days	0	++++	16	+0.2	0.1	+0.2	0.1

[1] Number of cases.
[2] Fisher, '36.

after 24 hours. The total rise amounted to 20% of the threshold before administration of pyridoxine. There was a slower but significant rise in threshold in the more severely deficient rats of group 5. In contrast to these, the rats in group 6 which had no evidence of deficiency except the excretion of high concentrations of xanthurenic acid showed no change after injection of the vitamin.

Table 2 summarizes the effects on electroshock threshold of tryptophane and glutamic acid added to the diet of rats. The mean change in electroshock threshold of each group is given in the 6th column. The 8th and 10th columns give the mean change in threshold 5 and 24 hours, respectively, after the administration of pyridoxine hydrochloride.

Each rat in groups 7 and 8 received slightly over 100 mg of DL-tryptophane daily. The addition of tryptophane to the deficient diet of group 7 resulted in a significant fall in the electroshock threshold. After the injection of pyridoxine there was a significant rise of threshold within 5 hours and a further rise by the end of 24 hours. On the other hand, the threshold of the control rats of group 8 failed to fall during the administration of tryptophane, or to rise in response to the injection of pyridoxine.

The addition of L-glutamic acid to the diet of mildly deficient rats appeared to raise the electroshock threshold. The animals of groups 9 and 10 were fed the basic deficient diet for 3 weeks, by the end of which time they showed signs of mild deficiency. To the diet of group 9 was added 2% of L-glutamic acid, while group 10 was continued on the basic deficient diet. By the end of 10 days the rats receiving glutamic acid showed a mean rise in electroshock threshold which was large enough to be significant, while the thresholds of the animals not receiving glutamic acid did not change. In more severely deficient rats the effect of glutamic acid was somewhat different. The 12 young rats of groups 11 and 12 were treated with desoxypyridoxine for 14 days. At the end of this time all had well-developed acrodynia and were excreting high concentrations of xanthurenic acid. Group 11 was then given glutamic acid in the basic deficient diet while group 12 was given the deficient diet alone. At the end of 6 days there was an insignificant drop in the electroshock threshold of both groups. The injection of pyridoxine then produced within 5 hours a highly significant rise of 2.5 mA in the threshold of group 11, whereas in group 12 it resulted in a rise of only 0.9 mA. Twenty-four hours after the injection of pyridoxine

TABLE 2

Effect of supplements to deficient diet

GROUP		ACRODYNIA	XANTHURENIC ACID	n [1]	EFFECT OF SUPPLEMENT ON ELECTROSHOCK THRESHOLD		EFFECT OF PYRIDOXINE ON ELECTROSHOCK THRESHOLD			
							5 Hours		24 Hours	
					Change mA	P [2]	Change mA	P	Change mA	P
7	Tryptophane in deficient diet, 5–6 days	++	+++	13	—1.4	0.01	+2.7	<0.01	+3.7	<0.01
8	Tryptophane in control diet, 5 days	0	0	6	+0.2	0.9	+0.2	0.8		
9	Glutamic acid in mild deficiency, 10 days	+	+++	10	+1.0	<0.05				
10	Controls for group 9. Plain deficient diet	+	+++	8	—0.2	0.7				
11	Glutamic acid in severe deficiency, 6 days	++++	+++	6	—0.4	0.1	+2.5	<0.01	+3.3	<0.01
12	Controls for group 11. Plain deficient diet	++++	+++	6	—0.5	0.2	+0.9	0.05	+2.5	<0.01

[1] Number of cases.

[2] Fisher, '36.

the total rise in electroshock threshold was 3.3 mA in the animals which had received glutamic acid and 2.5 mA in the controls.

Cellular hydration and electroshock threshold

It has been shown by Swinyard, Toman and Goodman ('46) that cellular hydration reduces the electroshock threshold of normal rats about 56% in a regular and predictable manner. The same is true of rats having mild to moderate pyridoxine deficiency. In 10 pyridoxine-deficient rats the mean electroshock threshold was 17 mA before and 8 mA after cellular hydration. Six control rats had a mean threshold of 20 mA before and 9 mA after hydration.

Xanthurenic acid and electroshock threshold

In order to determine whether any of the results given above could be explained on the basis of convulsant properties of xanthurenic acid, the effect of subcutaneous injections of this substance upon the electroshock thresholds of rats was investigated.[5] Ten normal rats were then given 1 mg of xanthurenic acid and 6 were given 2.5 mg in divided doses. At the end of 5 hours the electroshock thresholds were determined. The urines of the rats were examined for xanthurenic acid throughout the 5 hours and were found to be intensely positive within 1 hour; at the end of 5 hours the test in the urines of animals which received 2.5 mg was far more positive than was usually observed in the urines of our deficient rats. Although this would appear to indicate a higher blood concentration of xanthurenic acid than occurred in the pyridoxine-deficient rats, it produced no change in electroshock threshold. A single rat which was given a total dose of 5 mg of xanthurenic acid likewise showed no change in threshold, while another which received 10 mg had only a questionable slight lowering of the threshold.

[5] An authentic sample of xanthurenic acid was synthesized for us by Dr. W. J. Horton of the Department of Chemistry, University of Utah.

Maximal seizure patterns

The pattern of maximal electroshock seizures has been described by Toman, Swinyard and Goodman ('46) as consisting of a latent period, a period of tonic flexion, a period of tonic extension, a clonic phase which is frequently absent, and a period of post-seizure depression. The average time from the application of the shock to the beginning of the extensor phase is 2.8 seconds and from the application of the shock to the end of the extensor phase, 14 seconds. The post-seizure depression is seldom less than 2 or more than 4 minutes in length.

In several respects the effect of pyridoxine deficiency on maximal seizure pattern is similar to that of anticonvulsant drugs. Moderate deficiency was produced in 11 young rats by the administration of desoxypyridoxine for 10 days. All these animals had shown normal seizure patterns before treatment with desoxypyridoxine. After treatment the latent period was increased slightly and the flexor phase was lengthened to an average duration of 8.5 seconds. The extensor phase was absent in 5 of the 11 rats and was extremely brief in the others. In all cases the clonic phase was present and the post-seizure depression was of normal duration. Injected xanthurenic acid had no effect on the maximal seizure pattern.

Brain and blood analyses

In order to determine whether the increased susceptibility of pyridoxine-deficient rats to electrically induced seizures may be explained on the basis of alterations in the electrolyte content of blood and brain, the analyses summarized in table 3 were made.

There was no difference between the results obtained on control and deficient rats. The data on both brain and plasma electrolytes are in essential agreement with those which Swinyard ('47) obtained in normal well-fed rats.

A few analyses were made to determine the acid-base balance in control and vitamin B_6-deficient rats. The blood sam-

ples in this case were not pooled. In 3 control and 3 deficient rats the acid-base picture was almost identical: the pH was 7.32 and the extra fixed acid amounted to approximately 6 mM/l. This low degree of acidosis, which in the control rats was undoubtedly the result of chronic starvation, does not account for any changes in electroshock threshold (Davenport and Davenport, '48).

The blood lactic and pyruvic acid levels were also the same in control and pyridoxine-deficient rats.

TABLE 3

Analyses of brains and plasma of control and pyridoxine-deficient rats

	WATER		CHLORIDE		SODIUM		POTASSIUM	
	n[1]	gm per kg	n	mEq per kg	n	mEq per kg	n	mEq per kg
Control brains	7	785	7	33.4	3	44.9	4	98.1
Deficient brains	7	786	6	33.4	4	45.0	4	98.0
Control plasma	5	921	4	106.1	4	145.8	1	5.2
Deficient plasma	4	920	3	105.4	3	147.0	1	5.1

[1] Number of pooled samples.

DISCUSSION

Growing rats which are given a diet deficient in vitamin B_6 show a progressive diminution in the rate of weight gain and a tendency for the electroshock threshold to fall. Weight-paired controls fed limited amounts of an adequate diet exhibit the same changes. The lowering of threshold in the controls is undoubtedly due to the fact that these animals are subjected to partial starvation (Davenport and Davenport, '48). In deficient rats starvation is probably not a major cause of the lowering of threshold, although it may be a contributory factor.

The injection of pyridoxine causes a rapid and highly significant rise of electroshock threshold in mildly deficient rats but has no effect in control animals fed appropriately limited amounts of an adequate diet. This indicates that even a mild degree of pyridoxine deficiency causes an increase in

brain excitability. In more severely deficient rats the injection of pyridoxine raises the threshold more slowly. Presumably in this case the changes caused by the deficiency are more profound and therefore less easily reversed. The effect of pyridoxine in correcting brain excitability of mildly deficient rats is not necessarily associated with increased food intake, for the 5-hour rise in electroshock threshold was observed in rats deprived of food for that interval. Reactions involving pyridoxine must therefore have some specific effect in the maintenance of normal brain excitability.

Our data provide both negative and positive evidence of the means by which a mild degree of pyridoxine deficiency alters the excitability of the brain. The deficiency does not change the electrolyte pattern of the brain or the response of the brain to the stress of cellular hydration; it does not change the acid-base or electrolyte pattern of the blood; and it does not change blood lactic or pyruvic acid concentrations. All these factors are known to cause or accompany increased brain excitability, and it is possible that they may be among the causes of the spontaneous convulsions of advanced pyridoxine deficiency, but their participation in the effects of early pyridoxine deficiency can be ruled out. On the other hand, the results of feeding DL-tryptophane and L-glutamic acid suggest strongly that an interference with transamination is responsible for the effect of pyridoxine deficiency on brain excitability.

The addition of extra tryptophane to the vitamin B_6-deficient diet intensifies the signs of deficiency, especially the excretion of xanthurenic acid (Lepkovsky, Roboz and Haagen-Smit, '43); Miller and Baumann, '45). Xanthurenic acid is a product of tryptophane metabolism, and its concentration in the urine is an index of the extent to which tryptophane metabolism has been deranged by the vitamin deficiency. The brain excitability of deficient rats increases when tryptophane is added to their diet. Our data show that this is not due to a convulsant action of the extra xanthurenic acid produced and suggest that it may not be directly concerned with de-

ranged tryptophane metabolism, since rats during the first few days of treatment with desoxypyridoxine excrete large amounts of xanthurenic acid but do not show a change in electroshock threshold. Rather, it seems probable that the presence of extra tryptophane increases the competition for pyridoxine among the enzyme systems which require this vitamin, and increases brain excitability indirectly, possibly through an effect on the transaminase system.

The data obtained from the experiments in which extra glutamic acid was fed to vitamin B_6-deficient rats present stronger positive evidence for a correlation between transamination and brain excitability. Glutamic acid is known to play a key role in transamination (Cohen and Hekhuis, '41). Although the administration of glutamic acid to rats maintained on a normal diet does not change their brain excitability (Goodman, Swinyard and Toman, '46), it raises the electroshock threshold of rats mildly deficient in vitamin B_6. In severe deficiency it fails to raise the threshold but does hasten its return toward normal after the injection of pyridoxine. Although it is true that the deficient diet itself contains about 6% of glutamic acid in the casein, it is possible that only a small percentage of this is released from the casein molecule and made available to the transaminase system. In any case, the result of adding 2% of free glutamic acid must be due to a mass action effect. A simple explanation of our findings is that extra glutamic acid promotes a more efficient utilization of pyridoxine in the transaminase system and that maintenance of normal transamination is essential for those tests of normal brain function employed in this study.

SUMMARY

1. The electroshock threshold of rats mildly deficient in pyridoxine rises significantly within 5 hours after the rats are injected with pyridoxine.

2. The electroshock threshold of weight-paired control rats is not affected by the injection of pyridoxine.

3. Cellular hydration reduces the electroshock threshold to a similar extent in normal and pyridoxine-deficient rats.

4. The concentrations of water, sodium, potassium and chloride in the brain and blood plasma and of lactic and pyruvic acid in the blood are the same in pyridoxine-deficient and control rats. Both types of rat have a very mild degree of metabolic acidosis.

5. When extra tryptophane is added to the diet of rats mildly deficient in pyridoxine the signs of deficiency are intensified and there is a fall in electroshock threshold. This fall can be reversed within 5 hours by the injection of pyridoxine.

6. Xanthurenic acid, a product of tryptophane metabolism, when injected into normal rats has no effect on electroshock threshold or maximal seizure pattern.

7. Feeding extra glutamic acid to rats mildly deficient in pyridoxine causes the electroshock threshold to rise.

8. Rats more severely deficient in pyridoxine show a slower rise in electroshock threshold after the injection of pyridoxine, but if the animals have been fed extra glutamic acid for 6 days they respond to injected pyridoxine as rapidly as do mildly deficient rats.

9. It is suggested that the maintenance of normal transaminase activity is essential for those tests of normal brain activity employed in this study.

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CATARACTS DUE TO DEFICIENCIES OF PHENYLALANINE AND OF HISTIDINE IN THE RAT. A COMPARISON WITH OTHER TYPES OF CATARACTS [1]

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TWENTY-SEVEN FIGURES

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Cataracts which could be definitely attributed to a nutritional deficiency were first observed by Day, Langston and O'Brien ('31) in rats deficient in what was then known as vitamin G, although Salmon, Hays and Guerrant ('28) had earlier observed what might have been similar lenticular changes in studying experimental pellagra. It later became apparent that the cataracts were caused by a specific deficiency of riboflavin (Day, Darby and Langston, '37). Curtis, Hauge and Kraybill ('32) found that "rats receiving inadequate protein from either low corn protein or low protein of the basal ration in the absence of additions of tryptophane developed permanent blindness. This blindness was characterized by white opaqueness of the eye and loss of the characteristic colors of the eye, quite different from ophthalmia due to vitamin A deficiency." The addition of tryptophane to the diet prevented this blindness. From this description and the published photographs it seems apparent

[1] Preliminary reports of this work have been published (Sydenstricker, Schmidt and Hall, '47b; Bowles, Sydenstricker, Hall and Schmidt, '47).

that these authors had observed the cataracts resulting from tryptophane deficiency, as were later described by Totter and Day ('42).

That the adequacy of nutrition with respect to other amino acids and to protein may be of importance in the development of cataracts is indicated by several studies. Mitchell and Cook ('37) found that a protein deficiency definitely hastened the development in the rat of cataracts due to feeding high levels of galactose. In 4 rats fed a diet containing 10% of casein, Rezende and de Moura Campos ('42) observed bilateral cataracts. The parent rats had been fed the same deficient diet. According to Hall and Sydenstricker ('47) diets of this type are suboptimal with respect to several amino acids in addition to the more striking deficiency in methionine and cystine. Patch ('34) had previously described the occurrence of cataracts in the larva of Amblystoma tigrinum fed a highly purified milk-powder-casein diet. These could be prevented by adding cystine to the diet. Later, Patch ('43) observed cataracts in larva fed other proteins. These could not be prevented by supplementing the diet with cystine. Recently Ferraro and coworkers ('47) observed an early lens change consisting of vacuolation of the cortex, in rats fed a valine-deficient diet.

During an investigation of ocular changes in various deficiency states, we observed lenticular opacities in rats deficient in phenylalanine and in histidine. These observations led to the study reported here.

EXPERIMENTAL METHODS

Rats of a Wistar strain were used in this study. A description of the diets fed and the details of treatment of each group of rats are given in other sections of this paper. During the period of the experiment, biomicroscopic examinations of the eyes were made 3 or 4 times a week. In order to facilitate examination of the lens, usually the pupil of the right eye was dilated with a 0.5% solution of atropine sulfate

in 0.9% saline. Most of the deficient rats were allowed to die of the deficiencies. A few were changed to the control diet in an attempt to obtain regression of the lenticular changes. Also a few rats with some degree of lenticular changes were sacrificed in order that the lenses might be photographed.

On death or after killing the animal, the lenses were dissected out, immersed in saline and examined with a dissecting microscope. With the lenses immersed in saline and illuminated by 2 photo-flood lamps, photographs at a magnification of approximately 1 × were made on 35 mm panchromatic film. Usually a normal lens from a rat of approximately the same age was included for comparison.

All rats were kept in individual cages. Further details of care of animals and techniques are given in an earlier paper (Bowles and associates, '46).

RESULTS

Cataracts in phenylalanine deficiency

Since in the study of corneal changes in phenylalanine deficiency (Sydenstricker and co-workers, '47a), 1 of 11 rats was observed to develop a cataract, 25 more rats with 8 control animals from the same litters were placed, when 25 or 26 days of age, on the phenyalanine-deficient diet [2] described in that paper.

Definite lenticular changes were observed in all but 2 of the 25 rats on the phenylalanine-deficient diet in from 17 to 33 days (mean, 23 days). These changes consisted of a progressive lessening of the transparency of the lenses until

[2] The phenylalanine-deficient diet consisted of an amino acid mixture which supplied approximately 3 times the minimum requirement of each of the indispensable amino acids (except phenylalanine) in utilizable form, and the following: salt mixture 4 gm, cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, riboflavin 1.6 mg, thiamine hydrochloride 0.4 mg, calcium pantothenate 2 mg, pyridoxine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. By appropriate modification of the amino acid content of this formula, histidine, threonine, leucine, isoleucine and protein-deficient diets were prepared.

at the time of death, after 20–35 days on the diet, various degrees of opacity were observed. The earliest change was a slight haziness of the lens substance. Next, the lens star became more readily visible. This was followed by a separation of the most superficial fibers, and sometimes the epithelial layer and possibly the superficial fibers appeared rough and granular. Frequently superficial, irregular, glistening areas were present when the lens was first removed, which disappeared after the lens had been in saline a few minutes. No changes in the lens capsule were observed. Later, after the appearance of a refractile line between nucleus and cortex, there developed in the nucleus a relatively dense central opacity. Figures 1, 2 and 4 show successive degrees of the superficial lens changes and the appearance of the refractile line. Figure 3 shows an early stage in the development of the central opacity.

The most extreme degrees of lenticular change in the rats deficient in phenylalanine could be observed satisfactorily only after death of the animal and removal of the lens. In the advanced stages of the deficiency disease the cornea became sufficiently opaque (Sydenstricker and co-workers, '47a) to make obscure the exact degree of lenticular change at the time when some of these rats were placed on the control diet in an attempt to produce reversal of the lenticular changes. However, before observation of the lenses with the biomicroscope became unsatisfactory in these rats and in those which died at this stage, superficial changes had developed to a moderate or extreme degree and the formation of a nuclear cataract had begun.

Five rats which had developed cataracts due to phenylalanine deficieny were changed to the control diet in an attempt to induce regression of the lenticular changes. This resulted in a complete reversal of the changes in the lenses except that the refractile line separating the superficial and deep portions of the lenses persisted. The corneal changes appeared to be completely reversed upon biomicroscopic examination. However, upon microscopic examination of the

dissected corneas, it could be seen that empty capillaries were still present.

Cataracts in histidine deficiency

The lenticular changes resulting from histidine deficiency were followed in 21 of our Wistar strain rats. Thirteen of these were the rats used by Sydenstricker and associates ('47a) in a study of the corneal changes in histidine deficiency. For 15 of the rats on the histidine-deficient diet and 8 rats fed a control ration, the details of the diets,[3] care of the animals, and the techniques used were as described in the earlier paper. The remaining 6 histidine-deficient rats and 3 control animals from 2 litters were fed diets in which a histidine-deficient casein hydrolysate [4] was used. The deficient diet consisted of histidine-deficient casein hydrolysate 14.5 gm, DL-tryptophane 0.4 gm, DL-methionine 0.3 gm, cod liver oil 2 gm, cottonseed oil 3 gm, salt mixture [5] 4 gm, choline chloride 0.2 gm, riboflavin 1.6 mg, thiamine hydrochloride 0.4 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2 mg, and a sufficient amount of sucrose to make 100 gm. The control diet was identical except that it contained 0.5 gm of L-histidine monohydrochloride in place of that amount of sucrose.

In histidine deficiency the early clouding of the superficial lens substance (fig. 5) was followed by an apparent granular disintegration of the more superficial fibers (fig. 6). This obscured any widening of the sutures that may have occurred. The development of opacity in the deeper lens substance was usually preceded by the appearance of a refractile line separating the superficial and deep portions of the lens (figs. 5 and 8). The final central opacity frequently was first ob-

[3] See footnote 2, p. 279.

[4] The histidine-deficient casein hydrolysate was prepared as described by Conrad and Berg ('37). The diets in which it was used were adaptations of those used by Conrad and Berg.

[5] The salt mixture was that used by McKibben, Madden, Black and Elvehjem ('39).

served deep in the center of the lens (fig. 8), later spreading peripherally to the refractile line (fig. 9). Less often it was first seen just inside the refractile line (fig. 7) with subsequent extension deeper to the center of the lens. The resultant nuclear cataract was of variable size but usually of extreme density. Under the conditions of our experiment the lenticular opacities from histidine deficiency usually were of greater density than those which we observed to result from deficiency of phenylalanine, tryptophane or riboflavin. This was probably due to the fact that most of the histidine-deficient rats lived long enough for the cataracts to be fully developed, while the rats deficient in phenylalanine, or tryptophane or riboflavin usually died of the deficiency shortly after the lenticular opacities had begun to appear.

The lenticular changes due to histidine deficiency appeared after the rats had been fed the histidine-deficient diet from 10 to 34 days (mean 25 days); all of the rats on this diet, which lived long enough, developed cataracts.

Excepting 1 rat which died shortly after being placed on the deficient diet, the histidine-deficient rats lived from 34 to 61 days (mean 42 days) after being placed on the diet.

There were no significant differences between the lenticular changes observed in the rats on the 2 histidine-deficient diets. The control rats for the histidine-deficient casein hydrolysate diet developed no lenticular changes, though they did show some degree of corneal vascularization. This would indicate that this diet was not adequate in every respect.

When 3 rats with cataracts due to histidine deficiency were put on the control diet, the degree of reversal of lenticular changes depended on how far they had progressed. In any case the more superficial changes regressed. If a refractile line had formed separating the deeper and outer portion of the lens, this would persist after other lenticular changes had disappeared (fig. 11). This corresponds to what was observed in the regression of cataracts due to phenylalanine deficiency. When 2 deficient rats were returned to the

control diet after the central opacity had begun to form, although a reversal of other changes began immediately, the formation of the dense central cataract went to completion (figs. 10 and 12). Once formed, the dense central cataract did not regress.

Cataracts in tryptophane deficiency

In order to make comparable studies of cataracts due to tryptophane deficiency, 23 rats 21 to 27 days of age were placed on a tryptophane-deficient diet. Ten rats from the same litters were placed on a control diet. The tryptophane-deficient diet consisted of zein [6] 30 gm, L-histidine monohydrochloride 0.3 gm, L-lysine monohydrochloride 1.35 gm, DL-valine 0.8 gm, DL-threonine 0.3 gm, DL-methionine 0.3 gm, salt mixture [7] 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, calcium pantothenate 2 mg, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.4 mg, and a sufficient amount of sucrose to make 100 gm. The control diet was identical with this except that it contained 0.4 gm of DL-tryptophane in place of a like amount of sucrose.

With tryptophane deficiency the initial changes in the lenses were observed to consist of an apparent loosening and slight separation of superficial fibers, both anteriorly and posteriorly with widening of suture lines, and sometimes a radiating opacity of the superficial fibers, both anteriorly and posteriorly with widening of suture lines, and sometimes a radiating opacity of the superficial fibers around the lens star. Later a concentric, refractile line appeared similar to what was observed in lenses of rats deficient in phenylalanine and histidine. Within the central portion there appeared reduction in transparency varying from slight, diffuse haziness to dense, white opacity. Frequently the opacity of this cen-

[6] Obtained from the Corn Products Refining Co., New York, N. Y.

[7] See footnote 5, p. 281.

tral portion was more dense peripherally, giving a ring-shaped appearance to the cataract. The peripheral portion of the lens, outside the refractile line, showed changes varying from slight haziness to moderate, fine diffuse opacity. The lenticular changes which were observed in tryptophane deficiency are illustrated in figures 13, 14 and 15. Our observations correspond essentially to those made by Totter and Day ('42), Albanese and Buschke ('42) and other investigators.

Lenticular changes in deficiencies of protein and of amino acids

The lenses from several rats used in the investigation of another problem were dissected and examined for abnormalities. Eight of the rats were on a protein-free diet [8] as used by Hall and co-workers ('46), 7 on a threonine-deficient diet,[8] 1 on a leucine-deficient diet [8] and 9 on an isoleucine-deficient diet [8] as described by Sydenstricker and associates ('47a).

In all of the 8 rats which had been on a protein-free diet some forms of early lenticular changes were observed similar to those described above for phenylalanine deficiency. These consisted of haziness, visible separation of superficial fibers and widening of the sutures in varying degree.

The lenses from 4 of the 7 rats on the threonine-deficient diet and from the rat on the leucine-deficient diet showed similar changes.

The dissected lenses from the 9 rats on the isoleucine-deficient diet appeared to be normal except for a questionable widening of the suture line in the eyes of 1 animal.

These observations and the fact that a definite haziness of the lenses was seen with the biomicroscope in these and other amino acid deficiencies has led us to conclude that lenticular changes of some degree may result in the rat from deficien-

[8] See footnote 2, p. 279.

cies of protein or of any of the indispensable amino acids except arginine.

Cataracts in riboflavin deficiency

Twelve rats from 2 litters were placed, at weaning, on a riboflavin-deficient diet which contained vitamin-free casein [9] 20 gm, salt mixture [10] 4 gm, U.S.P. cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2 mg, thiamine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. In addition to the 12 rats used here, at various times we have made biomicroscopic examination of the lenticular changes occurring in other riboflavin-deficient rats which were available for such study. While there were no litter-mate controls for the 12 rats on the riboflavin-deficient diet, we have had at various times 71 rats which were fed for different periods this same diet but with added riboflavin. None of the 71 rats developed lenticular changes which could be observed with the biomicroscope.

In riboflavin deficiency the initial lenticular changes observed were a loosening and separation of superficial fibers and slight opening of sutures. Frequently, immediately after removal of the lens, there were seen in the loosened superficial fibers irregular areas of glistening, gray opacity which disappeared completely after the lens had been in saline a few minutes. This is similar to what was seen in phenylalanine-deficiency. The more advanced changes observed consisted of diffuse granular opacity in the superficial lens substance, the appearance of a refractile line between cortex and nucleus and development of a dense, white, spherical nuclear cataract. The lenticular changes observed in riboflavin-deficiency are illustrated in figures 17 and 18.

Our observations are similar to those made by Day, Langston and O'Brien ('31) with regard to this type of cataract.

[9] Labco brand.

[10] See footnote 5, p. 281.

Spontaneous cataracts

Out of some 1200 rats from our colony of Wistar strain animals, the eyes of which have been examined with the biomicroscope, in only 2 litters (8 and 5 rats, respectively) have spontaneous cataracts been observed. In the first litter cataracts were observed to appear almost simultaneously in 5 of the 8 rats when they were 104 days of age. The other 3 rats from the litter had previously been used in another experiment and killed. The cataracts were in the form of dense, cottonlike, nuclear opacities in otherwise apparently normal lenses. The size and density of the cataracts increased for 7 to 10 days at which time they varied from the size illustrated in figure 16 to approximately one-fourth of the diameter of the lens. Three of the animals were sacrificed for study and photographing of the lenses. The eyes of the remaining 2 rats were examined at periodic intervals for 51 and 112 days longer but no further lenticular changes took place.

The second litter of rats with spontaneous cataracts was the result of breeding 2 rats from a litter which consisted of runts. Of the 5 offspring, 4 were nearly normal in size, and the fifth, a runt. All 5 had cataracts when the eyes were first examined at 28 days of age. All except 1 had bilateral cataracts which were bilaterally quite unsymmetrical. Six of these cataracts, when first observed, were central nuclear cataracts of varying sizes which increased until the smallest were about the size of cataracts shown in figure 16 and the largest filled two-thirds of the diameter of the lens. Of 2 cataracts which were markedly eccentric when first observed, 1 spread across the lens to form a roughly spherical central cataract. The other spread around the circumference of the nucleus to form a ring-shaped cataract which eventually spread to the center. One of the cataracts which was ring-shaped when first observed, after forming radial white projections to the center of the lens, developed into a dense, white, central cataract. One eye of 1 rat appeared normal. The other eye of this rat contained the smallest and least

dense of the cataracts seen in this litter. Except for the nuclear opacities, the lenses all appeared to be normal. The cataracts developed rapidly for 7 to 14 days from the time they were first observed and then increased slightly in size and density during the next 6 weeks. Hereditary cataracts in the rat have been observed by Bourne and Grüneberg ('39) and by Lambert and Sciuchetti ('35).

Cataracts in a rat on a high tyrosine diet

Out of 73 rats of varying ages which were fed a 20% casein diet containing 10% additional L-tyrosine, 1 young adult rat developed large, dense, nuclear cataracts after 6½ months on this diet (fig. 27). None of the other rats, all of which had been on the diet for shorter periods of time, developed cataracts. Various organic compounds of the aromatic series, of which tyrosine is a member, are known to produce cataracts.

DISCUSSION

In general, there are marked similarities between the degenerative changes in the lens resulting from deficiencies of protein, amino acids or of riboflavin. These changes are: general haziness of the lens, separation of the superficial fibers, widening of the sutures, diffuse granular opacities in the cortex, the appearance of a refractile line separating cortex and nucleus, and dense nuclear opacity of variable extent.

The spontaneous cataracts seen in the 2 litters were similar in appearance but more irregular in outline and were not associated with the superficial changes seen in the nutritional deficiencies.

In general we have observed the development of corneal changes in protein, amino acid and riboflavin deficiencies to be bilaterally more uniform than were the lenticular changes. In table 1 a summary is given and a comparison is made of the corneal and lenticular changes in protein, amino acid and riboflavin deficiencies. This table shows for these defi-

control diet after the central opacity had begun to form, although a reversal of other changes began immediately, the formation of the dense central cataract went to completion (figs. 10 and 12). Once formed, the dense central cataract did not regress.

Cataracts in tryptophane deficiency

In order to make comparable studies of cataracts due to tryptophane deficiency, 23 rats 21 to 27 days of age were placed on a tryptophane-deficient diet. Ten rats from the same litters were placed on a control diet. The tryptophane-deficient diet consisted of zein [6] 30 gm, L-histidine monohydrochloride 0.3 gm, L-lysine monohydrochloride 1.35 gm, DL-valine 0.8 gm, DL-threonine 0.3 gm, DL-methionine 0.3 gm, salt mixture [7] 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, calcium pantothenate 2 mg, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.4 mg, and a sufficient amount of sucrose to make 100 gm. The control diet was identical with this except that it contained 0.4 gm of DL-tryptophane in place of a like amount of sucrose.

With tryptophane deficiency the initial changes in the lenses were observed to consist of an apparent loosening and slight separation of superficial fibers, both anteriorly and posteriorly with widening of suture lines, and sometimes a radiating opacity of the superficial fibers, both anteriorly and posteriorly with widening of suture lines, and sometimes a radiating opacity of the superficial fibers around the lens star. Later a concentric, refractile line appeared similar to what was observed in lenses of rats deficient in phenylalanine and histidine. Within the central portion there appeared reduction in transparency varying from slight, diffuse haziness to dense, white opacity. Frequently the opacity of this cen-

[6] Obtained from the Corn Products Refining Co., New York, N. Y.

[7] See footnote 5, p. 281.

tral portion was more dense peripherally, giving a ring-shaped appearance to the cataract. The peripheral portion of the lens, outside the refractile line, showed changes varying from slight haziness to moderate, fine diffuse opacity. The lenticular changes which were observed in tryptophane deficiency are illustrated in figures 13, 14 and 15. Our observations correspond essentially to those made by Totter and Day ('42), Albanese and Buschke ('42) and other investigators.

Lenticular changes in deficiencies of protein and of amino acids

The lenses from several rats used in the investigation of another problem were dissected and examined for abnormalities. Eight of the rats were on a protein-free diet [8] as used by Hall and co-workers ('46), 7 on a threonine-deficient diet,[8] 1 on a leucine-deficient diet [8] and 9 on an isoleucine-deficient diet [8] as described by Sydenstricker and associates ('47a).

In all of the 8 rats which had been on a protein-free diet some forms of early lenticular changes were observed similar to those described above for phenylalanine deficiency. These consisted of haziness, visible separation of superficial fibers and widening of the sutures in varying degree.

The lenses from 4 of the 7 rats on the threonine-deficient diet and from the rat on the leucine-deficient diet showed similar changes.

The dissected lenses from the 9 rats on the isoleucine-deficient diet appeared to be normal except for a questionable widening of the suture line in the eyes of 1 animal.

These observations and the fact that a definite haziness of the lenses was seen with the biomicroscope in these and other amino acid deficiencies has led us to conclude that lenticular changes of some degree may result in the rat from deficien-

[8] See footnote 2, p. 279.

cies of protein or of any of the indispensable amino acids except arginine.

Cataracts in riboflavin deficiency

Twelve rats from 2 litters were placed, at weaning, on a riboflavin-deficient diet which contained vitamin-free casein [9] 20 gm, salt mixture [10] 4 gm, U.S.P. cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2 mg, thiamine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. In addition to the 12 rats used here, at various times we have made biomicroscopic examination of the lenticular changes occurring in other riboflavin-deficient rats which were available for such study. While there were no litter-mate controls for the 12 rats on the riboflavin-deficient diet, we have had at various times 71 rats which were fed for different periods this same diet but with added riboflavin. None of the 71 rats developed lenticular changes which could be observed with the biomicroscope.

In riboflavin deficiency the initial lenticular changes observed were a loosening and separation of superficial fibers and slight opening of sutures. Frequently, immediately after removal of the lens, there were seen in the loosened superficial fibers irregular areas of glistening, gray opacity which disappeared completely after the lens had been in saline a few minutes. This is similar to what was seen in phenylalanine-deficiency. The more advanced changes observed consisted of diffuse granular opacity in the superficial lens substance, the appearance of a refractile line between cortex and nucleus and development of a dense, white, spherical nuclear cataract. The lenticular changes observed in riboflavin-deficiency are illustrated in figures 17 and 18.

Our observations are similar to those made by Day, Langston and O'Brien ('31) with regard to this type of cataract.

[9] Labco brand.

[10] See footnote 5, p. 281.

Spontaneous cataracts

Out of some 1200 rats from our colony of Wistar strain animals, the eyes of which have been examined with the biomicroscope, in only 2 litters (8 and 5 rats, respectively) have spontaneous cataracts been observed. In the first litter cataracts were observed to appear almost simultaneously in 5 of the 8 rats when they were 104 days of age. The other 3 rats from the litter had previously been used in another experiment and killed. The cataracts were in the form of dense, cottonlike, nuclear opacities in otherwise apparently normal lenses. The size and density of the cataracts increased for 7 to 10 days at which time they varied from the size illustrated in figure 16 to approximately one-fourth of the diameter of the lens. Three of the animals were sacrificed for study and photographing of the lenses. The eyes of the remaining 2 rats were examined at periodic intervals for 51 and 112 days longer but no further lenticular changes took place.

The second litter of rats with spontaneous cataracts was the result of breeding 2 rats from a litter which consisted of runts. Of the 5 offspring, 4 were nearly normal in size, and the fifth, a runt. All 5 had cataracts when the eyes were first examined at 28 days of age. All except 1 had bilateral cataracts which were bilaterally quite unsymmetrical. Six of these cataracts, when first observed, were central nuclear cataracts of varying sizes which increased until the smallest were about the size of cataracts shown in figure 16 and the largest filled two-thirds of the diameter of the lens. Of 2 cataracts which were markedly eccentric when first observed, 1 spread across the lens to form a roughly spherical central cataract. The other spread around the circumference of the nucleus to form a ring-shaped cataract which eventually spread to the center. One of the cataracts which was ring-shaped when first observed, after forming radial white projections to the center of the lens, developed into a dense, white, central cataract. One eye of 1 rat appeared normal. The other eye of this rat contained the smallest and least

dense of the cataracts seen in this litter. Except for the nuclear opacities, the lenses all appeared to be normal. The cataracts developed rapidly for 7 to 14 days from the time they were first observed and then increased slightly in size and density during the next 6 weeks. Hereditary cataracts in the rat have been observed by Bourne and Grüneberg ('39) and by Lambert and Sciuchetti ('35).

Cataracts in a rat on a high tyrosine diet

Out of 73 rats of varying ages which were fed a 20% casein diet containing 10% additional L-tyrosine, 1 young adult rat developed large, dense, nuclear cataracts after 6½ months on this diet (fig. 27). None of the other rats, all of which had been on the diet for shorter periods of time, developed cataracts. Various organic compounds of the aromatic series, of which tyrosine is a member, are known to produce cataracts.

DISCUSSION

In general, there are marked similarities between the degenerative changes in the lens resulting from deficiencies of protein, amino acids or of riboflavin. These changes are: general haziness of the lens, separation of the superficial fibers, widening of the sutures, diffuse granular opacities in the cortex, the appearance of a refractile line separating cortex and nucleus, and dense nuclear opacity of variable extent.

The spontaneous cataracts seen in the 2 litters were similar in appearance but more irregular in outline and were not associated with the superficial changes seen in the nutritional deficiencies.

In general we have observed the development of corneal changes in protein, amino acid and riboflavin deficiencies to be bilaterally more uniform than were the lenticular changes. In table 1 a summary is given and a comparison is made of the corneal and lenticular changes in protein, amino acid and riboflavin deficiencies. This table shows for these defi-

TABLE 1

Summary of corneal and lenticular changes observed in young rats fed diets deficient in protein, amino acids or riboflavin[1]

DEFICIENCY	CORNEA			LENS				
				Superficial changes			Deep changes	
	Vascularization	Opacity	Superficial ulceration	Haziness	Separation of fibers	Widening of sutures	Line separating cortex and nucleus	Nuclear opacity
Protein	++++	+++	+	+	+	+		
Methionine	+++	+		+				
Phenylalanine	++++	++++	+	+	+	+	+	+
Leucine	+++	+++		+	+	+		
Isoleucine	++	++		+				
Arginine	+	+						
Histidine	++	++		+	+	+	+	+
Threonine	++	++		+	+	+		
Valine	++	++		+				
Lysine	+++	++		+				
Tryptophane	++++	++++	+	+	+	+	+	+
Riboflavin	+++	++++		+	+	+	+	+

[1] The corneal changes summarized in this table were described by Hall et al. ('46), Berg and co-workers ('47), Sydenstricker and associates ('47a) and Bowles et al. ('46). The above estimates of the degree of corneal changes in specific deficiencies take into account the extent and severity of the particular change, the rapidity of its development, and the length of time the rat lived after the change first appeared.

ciencies a similarity between the extent and severity of the changes in the cornea and those in the lens, but with the exception that in histidine deficiency the lenticular changes are more extreme.

It is of interest to note that Mitchell ('36) found Wistar strain rats, such as were used in this study, to be more resistant than some other strains to the development of cataracts due to the feeding of high levels of lactose or galactose.

No lenticular changes were observed in any of the control rats used in this study.

The weight changes of the rats on the experimental diets in this study were essentially the same as those reported by Sydenstricker et al. ('47a) and Bowles and co-workers ('46) where similar diets were used.

Day, Darby and Cosgrove ('38) were able by the administration of riboflavin to arrest the development of the dense central cataracts in some riboflavin-deficient rats. In the present study the number of rats deficient in phenylalanine and in histidine in which an attempt was made to produce a regression of lenticular changes was too small to determine conclusively whether such an arrest was possible.

The routine use of atropine sulfate to dilate the pupils of the right eyes of the rats used in this study resulted in a more extreme keratitis of the cornea than in the left eyes of these rats where atropine was used only occasionally, or than in the eyes of other rats on the same diets where biomicroscopic examinations were made as frequently but where the pupils were not dilated. The use of atropine had no effect on the development of the cataract.

SUMMARY

The lenticular changes observed in rats deficient in phenylalanine, histidine, tryptophane or riboflavin were: general haziness, separation of the superficial fibers, widening of the sutures, diffuse and granular opacities in the cortex, granular degeneration of the superficial cortex, the appearance of a refractile line separating cortex and nucleus, and

dense nuclear opacity of variable extent. The characteristic changes in each deficiency are described and illustrated. Lenticular changes of some degree were observed in deficiency of protein and of each of the indispensable amino acids except arginine. When rats deficient in phenylalanine or histidine were returned to the control diet, a reversal of superficial changes resulted, though the dense nuclear opacities were found to be irreversible.

The spontaneous cataracts observed in 2 litters of rats, and cataracts in a rat on a high tyrosine diet are also described.

ACKNOWLEDGMENTS

This study was aided by grants from the John and Mary R. Markle Foundation and from the Division of Grants, National Institute of Health, U. S. Public Health Service. Some of the amino acids used in this study were donated by Winthrop Chemical Company. We thank Nathaniel Thornton, II, for assistance, and Elizabeth Thompson for care of the animals.

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PLATE 1

EXPLANATION OF FIGURES

(All 7 ×, reduced approximately three-eighths)

Photographs of lenses dissected from rats which had been on normal diet and on deficient diets. In each are the 2 lenses from 1 rat with a normal lens in the center except in 9 where a normal lens is not included and in 10 where the normal lens is at the left.

1 through 4 Phenylalanine-deficient diet for 46, 36, 30 and 31 days, respectively.

5 through 9 Histidine-deficient diet for 34, 45, 37, 34 and 62 days, respectively.

10, 11 and 12 Histidine-deficient diet for 31, 58 and 40 days and control diet for 21, 27 and 22 days, respectively.

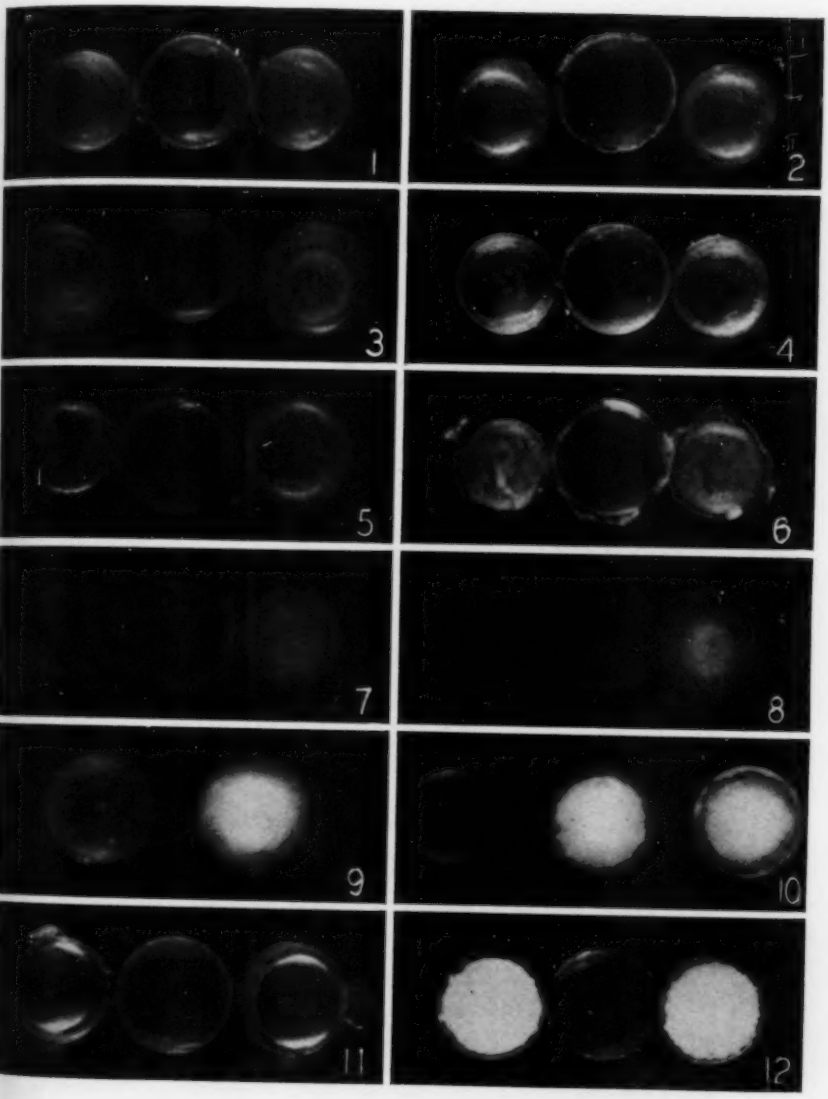
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PLATE 2

EXPLANATION OF FIGURES

(All 7 ×, reduced approximately three-eighths)

13 through 18 Photographs of lenses dissected from rats which had been on normal diet and on deficient diets. In each are the 2 lenses from 1 rat with a normal lens in the center except in 13 where a normal lens is not included.

13, 14 and 15 Tryptophane-deficient diet for 23, 62 and 51 days, respectively.

16 Spontaneous cataracts, observed in the rat when 104 days of age. Rat killed and photographed 7 days later.

17 and 18 Riboflavin-deficient diet for 119 and 104 days, respectively.

19 through 26 Photographs of eyes of rats which had been on normal diet and on deficient diets. The 2 highlights in each are reflections of the photoflood lamps and should be disregarded.

19 Histidine-deficient diet for 45 days. The lens from this eye is in 6.

20 Normal diet.

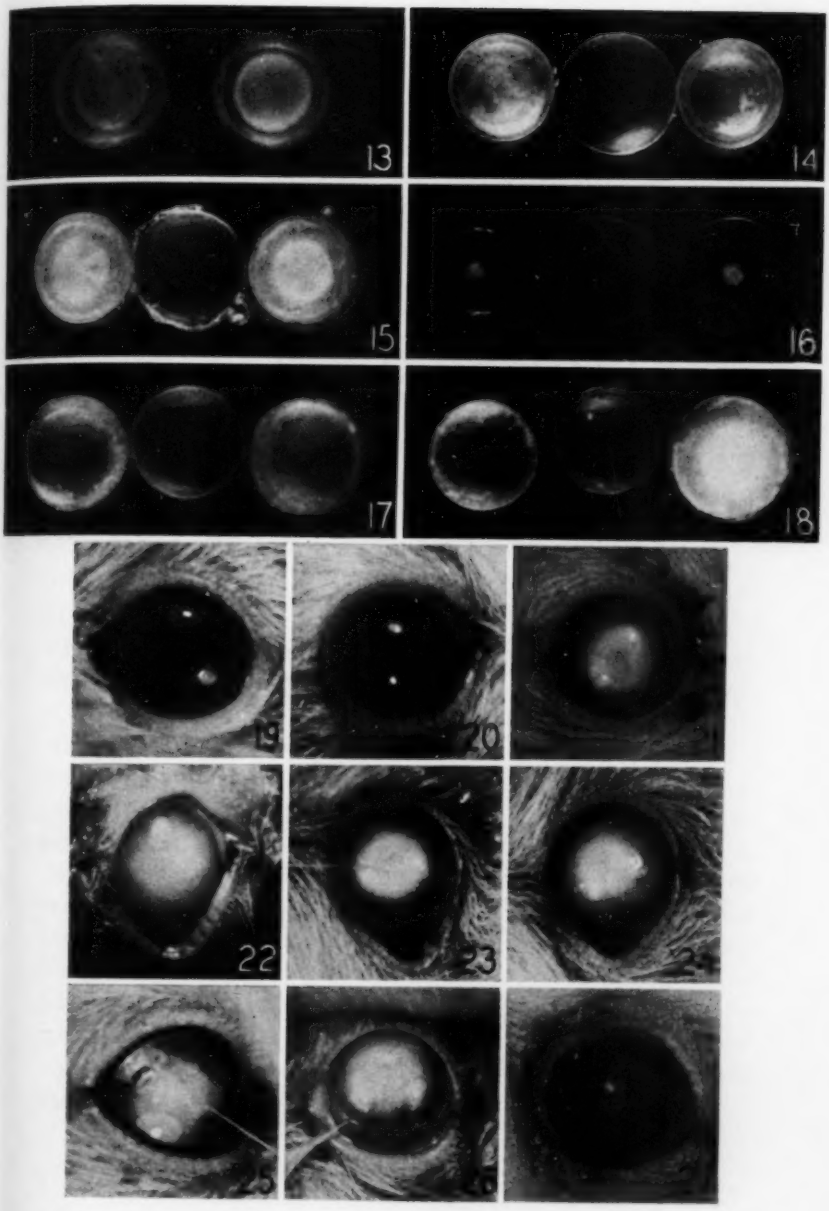
21 and 22 Histidine-deficient diet for 34 and 62 days. The lenses from these eyes are on the right in 8 and 9.

23 and 24 Histidine-deficient diet 31 days, and control diet 21 days. The lenses from these eyes are in 10.

25 Histidine-deficient diet 40 days and control diet 22 days. The lens from this eye is in 12.

26 Tryptophane-deficient diet for 23 days. The lens from this eye is on the right in 13.

27 Eye of a rat on high-tyrosine diet for 196 days. (The vessels of cornea and iris show injection with India ink. The photograph of the eye of a normal control rat which had been similarly injected was indistinguishable from the photograph of the normal uninjected eye shown in figure 20).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THE RELATION OF PYRIDOXINE TO THE GROWTH OF CHICKS FED RATIONS CONTAINING LINSEED OIL MEAL

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TWO FIGURES

(Received for publication March 24, 1948)

Numerous workers have demonstrated that linseed oil meal is detrimental to growing chickens when it is fed at levels of 4.5% or more of the ration (Bethke et al., '28; Ackerson et al., '38; Sherwood and Couch, '40; Slinger et al., '43; Hammond, '44; Heuser and Norris, '44; Heuser et al., '46). However, the nutritional value of linseed oil meal for promoting growth in chicks may be improved by treating the meal with water (Kratzer, '46; McGinnis and Polis, '46; Kratzer, '47; MacGregor and McGinnis, '48). It has also been shown that improvement approximately equal to that observed following the water treatment can be produced by the addition of a mixture of synthetic vitamins to a ration containing untreated linseed oil meal (Kratzer and Williams, '48). The problem has been studied further in an attempt to determine the relationship of the addition of various vitamins to the improvement in growth and to elucidate the mechanism of the changes brought about by the water treatment.

PROCEDURE AND RESULTS

Methods

Single Comb White Leghorn chicks were fed a standard starting ration for a period of about 10 to 14 days, after which they were divided into groups and were fed the experimental rations. The chicks were kept in electrically heated batteries with raised wire floors and were supplied with feed and water ad libitum. The experiments were continued for 18 to 20 days.

The experimental rations contained the following: crude casein, 5.0 gm; dried whey, 10.0 gm; alfalfa meal, 10.0 gm; steamed bone meal, 1.0 gm; cottonseed oil, 5.0 gm; ground limestone, 2.0 gm; salt, 1.0 gm; manganese sulfate, 0.0125 gm; fish oil (1000 A, 400 D), 0.125 gm; ground wheat, 20.0 gm; ground barley, 16.0 gm and either untreated or water-treated linseed oil meal 30.0 gm. The linseed oil meal used [1] was an experimentally produced, solvent-processed sample differing from the old-process linseed oil meal used in previous work. The vegetable oil was found essential to prevent the feed from sticking to the beaks of the chicks. The vitamin mixture which was fed to certain groups supplied per kilogram of diet: thiamine hydrochloride 20 mg; riboflavin 25 mg; pyridoxine hydrochloride 35 mg; calcium pantothenate 110 mg; nicotinic acid 200 mg; pteroylglutamic acid [2] 10 mg; inositol 4 gm and choline 4 gm. In the second experiment biotin [3] was included in the mixture at the level of 0.2 mg per kilogram. When additions of single vitamins were made, they were at the above levels. The water treatment of the linseed oil meal was carried out by mixing the meal with 3 times its

[1] Kindly donated by Archer-Daniel-Midland Company, Minneapolis, Minn., through the courtesy of Dr. J. W. Hayward.

[2] Kindly donated by Lederle Laboratories, Inc., Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

[3] Kindly donated by Merck and Company, Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

weight of water and allowing it to stand for 1 day at room temperature. The material was then dried at approximately 65°C. and ground in a burr mill.

A. Qualitative study of vitamin supplementation of linseed oil meal

In the first experiment the effect of the addition of various vitamins to the ration containing untreated linseed oil meal

TABLE 1

The effect upon the growth of chicks of water treated linseed oil meal and/or adding vitamins

SUPPLEMENT	AVE. GAIN IN WEIGHT (GM.)	
	Trial A	Trial B
Experiment 1 — Trial A, 18 days; B, 20 days		
Linseed oil meal	34	42
Linseed oil meal + vitamin mix	118	198
Linseed oil meal + pyridoxine	86	181
Linseed oil meal + inositol	53	159
Linseed oil meal + choline	70	
Linseed oil meal + pyridoxine + inositol	92	
Linseed oil meal + pyridoxine + inositol + choline	105	
Water-treated linseed oil meal	78	171
Water-treated linseed oil meal + vitamin mix	128	
Experiment 2 — 19 days		
Linseed oil meal	97	
Linseed oil meal + pyridoxine	166	
Linseed oil meal + vitamin mix	169	
Linseed oil meal + inositol	105	
Water-treated linseed oil meal	180	
Water-treated linseed oil meal + pyridoxine	188	
Stock mash	191	

was tested. The vitamin additions which were made and the results obtained are shown in table 1. The addition of the vitamin mixture caused the greatest increase in growth. Pyridoxine alone caused a large increase in growth, but in both trials this was not equal to the growth produced by the

vitamin mix. Inositol caused a slight increase in growth when it was added to the ration containing no other vitamin supplements, but caused no response in 2 trials when it was added to rations supplemented with pyridoxine. Choline caused an increase in growth when it was added to the ration either alone or in combination with pyridoxine and inositol. The water treatment of linseed oil meal caused a growth increase approximately equal to that caused by the addition of pyridoxine, but it was not as great as the increase caused by the vitamin mixture. These data were interpreted to indicate that the water treatment increased growth by eliminating the need for added pyridoxine, but the water-treated linseed oil meal ration was still suboptimal with respect to choline. This deficiency of choline may have resulted from the solvent extraction process while the old-process linseed oil meal used in earlier work was adequate in choline.

To test the supposition that the water treatment resulted in an improvement of linseed oil meal for chicks by virtue of eliminating the necessity for supplying pyridoxine to the ration, a second experiment was conducted in which the basal ration contained additions of the B-complex vitamins. The basal ration was similar to that originally described with the exception of the following additions: choline 0.1 gm; inositol 0.1 gm; thiamine hydrochloride 0.1 mg; riboflavin 0.2 mg; calcium pantothenate 0.6 mg; nicotinic acid 0.9 mg; biotin 0.005 mg and pteroylglutamic acid 0.05 mg per 100 gm. The groups included in the trial are shown in table 1. Marked improvement in growth occurred when pyridoxine was added to the untreated linseed oil meal; none occurred when it was added to the water-treated meal. There was no improvement beyond that caused by the addition of pyridoxine when the vitamin mixture was added. The addition of inositol caused no appreciable response; however, the basal ration in this trial contained inositol. The data indicate that the major part of the response, if not all, resulting from the water treatment of linseed oil meal was due to eliminating the need for added pyridoxine.

B. Quantitative relationship of linseed oil meal and pyridoxine

Since it was clearly indicated that pyridoxine alleviated the growth inhibiting properties of linseed oil meal, it seemed desirable to determine the amount of pyridoxine required to prevent the detrimental effects. A basal ration was used which contained 30% solvent-processed linseed oil meal and was similar to that used in the second experiment of part A.

Varying amounts of pyridoxine hydrochloride were added to this ration. Ten chicks per group were continued on these

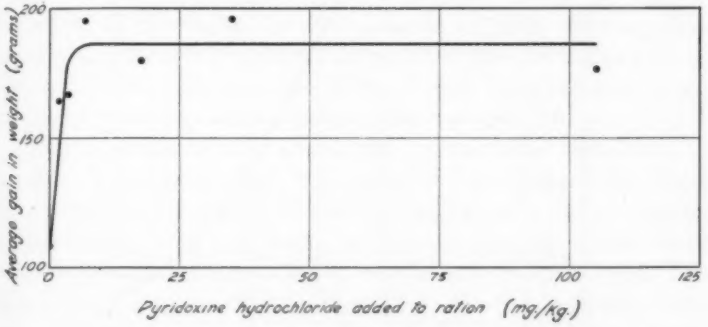

Fig. 1 Relation of the level of pyridoxine hydrochloride added to a ration containing linseed oil meal to the growth of chicks.

rations 20 days. The results are shown in figure 1. Maximum growth was obtained with the addition of 7 mg per kilogram, while 1.75 and 3.5 mg per kilogram produced growth intermediate between the 7 mg level and the group receiving only the basal ration. This was assayed for pyridoxine by the use of Neurospora sitophila [4] (Stokes, '47) and was found to contain 12.5 mg per kilogram.

In other groups of the same experiment no supplementary pyridoxine was added to the ration but the level of linseed oil meal was varied from 0 to 30%. The crude protein content

[4] Kindly donated by Dr. G. W. Beadle, California Institute of Technology, Pasadena.

of the rations was maintained constant by the addition of soybean oil meal and ground wheat as the linseed oil meal was reduced. The growth data are presented in figure 2. A reduction in growth was not apparent until the level of linseed oil meal in the ration exceeded 10%, but the reduction was very rapid as the linseed oil meal was increased beyond this level. The curve is admittedly subject to some error due to

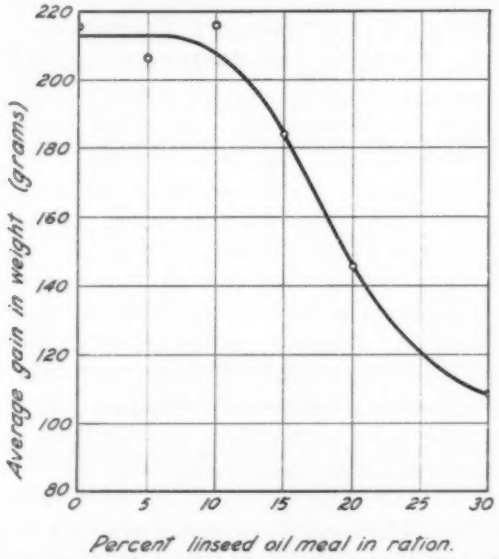

Fig. 2 Relation of the level of linseed oil meal in the ration to the growth of chicks.

variations in the amino acid content of the rations as the preparations of linseed, soybean and wheat proteins varied, but it is felt that the greatest differences were due to the specific growth depressing action of linseed meal. This is substantiated by the fact that groups receiving 30% linseed oil meal and supplemented with adequate pyridoxine grew nearly as rapidly as those receiving the low levels of linseed oil meal.

DISCUSSION

The data indicate that linseed oil meal depressed growth of chicks by rendering the ration deficient in pyridoxine. However, the total pyridoxine in the ration (12.5 mg/kg) was many times the level of 2.0 to 3.0 mg per kilogram which has been reported to be required by chicks for normal growth in rations which contain no linseed oil meal (Briggs et al., '42; Kratzer et al., '47). The principal effect of the water treatment of linseed oil meal was to reduce the requirement for pyridoxine. The linseed oil meal itself contained approximately 18 mg of pyridoxine per kilogram. Thus, the amount supplied to the ration by 30% linseed oil meal was approximately 5.4 mg per kilogram. It can therefore be assumed that the non-linseed components of the ration supplied over 7 mg of pyridoxine to each kilogram of the ration.

The mechanism by which the pyridoxine deficiency is created is not apparent. There are at least 2 ways by which this could occur. First, if linseed contained a factor capable of combining with pyridoxine to form a complex which would render pyridoxine unavailable, a deficiency would result. This would be analogous to the action of avidin in rendering biotin unavailable and thereby creating a deficiency of biotin (Eakin et al., '40). In such a situation the water treatment would have to destroy the pyridoxine-combining compound so that it could not form a complex.

A second theory for the apparent pyridoxine deficiency involves the presence in linseed oil meal of a compound which is similar enough to pyridoxine to substitute for it in biologically important complexes, but different enough so that it does not possess biological activity, thus making it a competitive inhibitor. Many such analogues have been recognized (Woolley, '47) but for the most part they have not been found in natural products. In such a theory the water treatment would be expected to destroy the competitive inhibitor or inactive analogue so it could not compete with pyridoxine. The addition of pyridoxine would replace that rendered inactive by the

supposed competitive inhibitor and provide the biologically active pyridoxine needed.

The fact that previous workers observed growth reduction at levels of linseed oil meal of 4.5% and higher while in the present work 10% seemed to be the critical level, probably reflects differences in the pyridoxine content of the basal rations used rather than differences in response. It is probable that our ration contained more pyridoxine than the former rations and it required a higher level of linseed to create an apparent pyridoxine deficiency.

The exact mechanism for the creation of an apparent pyridoxine deficiency in chicks by feeding linseed oil meal and the processes by which linseed oil meal can be altered to prevent this action by water treatment must await further experimentation.

SUMMARY

When linseed oil meal was fed to chicks at 30% of the ration an apparent pyridoxine deficiency resulted, although the ration contained many times the amount of pyridoxine normally required by chicks. The deficiency was prevented by adding synthetic pyridoxine or by treating the linseed oil meal with water.

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ADDENDUM

Subsequent determinations of the pyridoxine content of linseed oil meal by the Neurospora sitophila method have shown considerable variations. Chemical analyses made by Mr. W. R. Flach of the Eastern States Farmer's Exchange, Buffalo, New York, indicated that both untreated and water-treated linseed oil meal contained approximately 6 mg of pyridoxine per kg.

There was no increase in pyridoxine resulting from the water treatment, and the amount present in the ration as found by either method was many times the required level.

THE RELATION BETWEEN URINARY EXCRETION AND TISSUE CONCENTRATIONS OF THIAMINE IN RATS

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ONE FIGURE

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Although the thiamine concentration of tissues has been studied by a number of workers (Westenbrink, '32; Brodie and McLeod, '35; Leong, '37; Schultz et al., '39; Ferrebee et al., '42; Mitchell and Isbell, '42; Sure and Ford, '42; Sarett and Perlzweig, '43; Schweigert et al., '43) under varying conditions, little attempt has been made to correlate tissue levels with urinary excretion. Since clinical evaluation of deficiency situations is in large part dependent upon urinary excretion data, it is of obvious importance to interpret such data in terms of what is going on in the tissues. The present experiments were undertaken to fill this gap.

EXPERIMENTAL

Plan

The general plan was to deplete a group of rats with respect to thiamine, sacrificing representatives of the group at intervals for tissue analysis and following the total urinary

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output of thiamine during the depletion process. Particular interest is attached to the state of the tissues when the "point of minimum excretion" in the urine was attained, since it has been postulated (Holt and Najjar, '43) that excretion above this level corresponds to a surplus above physiological needs and depletion beyond this point to potential physiological deficiency.

Animals

The animals employed were adult males from a colony of mixed albino and hooded Norwegian rats. They were kept in individual metabolism cages to permit collection of urine.

Diet

After a period on the standard McCollum diet the animals were placed on a thiamine deficient diet identical with that described in a previous publication (Najjar and Holt, '43) with the exception that sucrose was employed in place of dextrimaltose.

Analytical methods

Urine was preserved with acetic acid and analyzed for thiamine by a modification (Najjar and Ketron, '44) of the standard thiochrome procedure (Hennessy and Cerecedo, '39). Analyses of free and combined thiamine (cocarboxylase) were carried out on brain, heart, kidney, liver and muscle. For free thiamine a sample of fresh tissue was macerated and extracted with boiling 2% acetic acid for 20 minutes. It was then centrifuged and the supernatant fluid decanted for thiamine analysis. For total thiamine the acetic acid extract, prepared as described above, was treated with takadiastase, the pH adjusted to 4.5 with alkali and incubated at 45°C. for 1 hour with occasional shaking. This was followed by centrifugation at high speed, then by decanting and analysis of the supernatant fluid. The combined thiamine was determined by difference.

RESULTS

Two groups of rats were studied. The first group of 12 rats served essentially as an orientation experiment to determine how long it would take for the urinary excretion of thiamine to fall to the minimum point and at what intervals rats should be sacrificed for tissue analysis. It was found that from 6 to 16 days were required for the urine to reach the minimum level in individual animals; the group as a whole

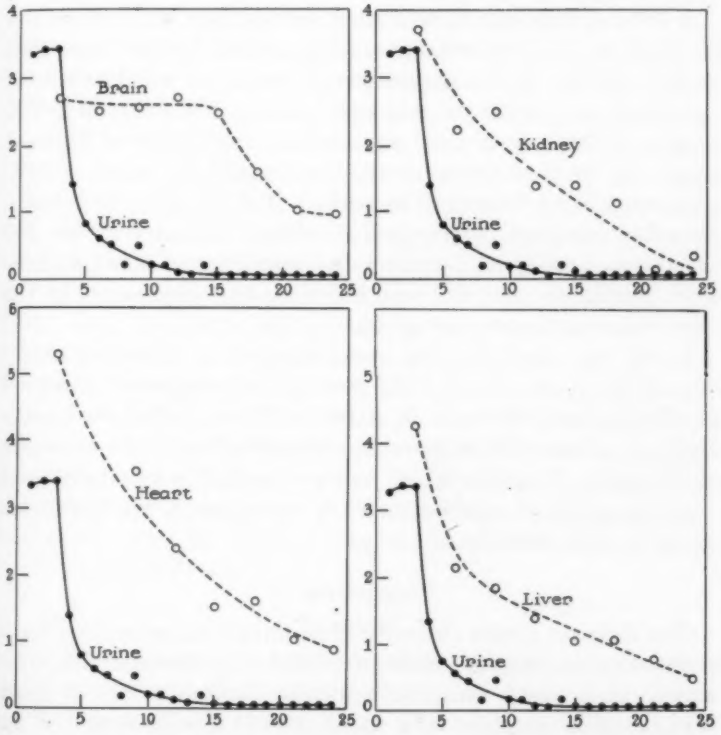

Fig. 1 Relation between thiamine of tissues and thiamine excretion in urine. Ordinates represent micrograms thiamine per gm dry tissue and micrograms per day of thiamine in urine. Abscissae represent days on thiamine deficient diet. The tissue figures represent averages of 4 animals sacrificed. The urine figures represent averages of surviving animals.

maintained a minimum excretion from the 17th day onward. Assays of tissues were made on animals sacrificed on the 9th and on the 31st day of the deficient diet. These assays were obviously not suitable for any correlation of tissue changes with the point of minimum excretion, but they were of interest in showing that virtually all of the tissue thiamine was combined, and that the combined thiamine of the brain (in contrast to other tissues) was maintained at least up to the 9th day although it fell thereafter.

A second experiment was then carried out with 32 rats, 4 of which were sacrificed for analysis every 3 days beginning on the 3rd day of the experimental period. Pooled urines of the surviving rats were assayed for thiamine each day. The results of this experiment are shown graphically in figure 1 where the total thiamine of the tissues and the urinary thiamine are shown through the period of developing deficiency. Separate analyses of free and combined thiamine of the tissues were carried out, but since the quantities of free thiamine were negligible and did not influence the picture, only the total thiamine figures are given.

It will be noted that the concentration of thiamine in the liver, kidney and heart falls steadily throughout the period of developing deficiency in a curve which shows no breaks and is of a logarithmic type. In sharp contrast is the behavior of the brain thiamine which is sustained at a constant level until the point of minimal urinary excretion is reached after which it falls sharply.

DISCUSSION

The data on tissue thiamine concentrations which we have reported above are in good agreement with those in the literature. It is clear that the thiamine concentration of most organs varies directly with the diet over a wide range of intakes, although there appears to be an upper limit (Schultz et al., '39) beyond which the tissue concentration cannot be increased. The tendency of the brain to maintain its thiamine concentration in the face of a reduced intake has also been

reported by others (Westenbrink, '32; Brodie and McLeod, '35). We have been able to find simultaneous determinations of tissue thiamine and urinary thiamine only in the work of Ferrebee et al. ('42), but these authors, although they compared normal animals with those on a deficient diet, did not study the progress of the deficiency changes in the tissues and urine or attempt a correlation between them.

It would appear from the data at hand that the brain differs from other tissues studied in not being able to store surplus thiamine. The sudden break in the concentration of brain thiamine when depletion reaches the point of minimum urinary excretion suggests the development of unphysiological changes in this tissue and is in keeping with the known susceptibility of the nervous system to thiamine deficiency. That the "point of minimum excretion" of thiamine in the urine is compatible with health and is not far above a level at which deficiency symptoms make their appearance is indicated by studies made by some of us of experimental thiamine deficiency in adult and adolescent humans (Najjar and Holt, '43; Holt, '44). Further studies made on infants (Holt et al., '46; Snyderman et al., to be published), although not carried to the point of deficiency symptoms, have shown that an intake which maintains the infant at the point of minimum excretion may be continued for long periods without evidences of clinical deficiency. Since the point of mimimum excretion can be readily determined, it provides what appears to be a highly useful end point for determining thiamine requirements under various circumstances.

SUMMARY

1. The thiamine content of rat tissues has been studied under conditions of developing thiamine depletion and has been correlated with the excretion of thiamine in urine.

2. Unlike other tissues studied the brain maintains its thiamine concentration in the face of a deficit of thiamine for a considerable period, after which there occurs an abrupt fall in thiamine content.

3. The critical point at which the brain begins to lose its thiamine corresponds to the attainment of the minimum level of urinary thiamine excretion. This finding supports the view that the point of minimum urinary excretion is of physiological significance.

4. It is suggested that the point of minimum excretion in the urine is a highly useful criterion for measuring thiamine requirements under various conditions.

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VITAMIN STUDIES IN MIDDLE-AGED AND OLD INDIVIDUALS

I. THE VITAMIN A, TOTAL CAROTENE AND $\alpha + \beta$ CAROTENE CONCENTRATIONS IN PLASMA [1]

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ONE FIGURE

(Received for publication February 24, 1948)

INTRODUCTION

In spite of the existence of an extensive literature dealing with the clinical aspects of vitamin A, data on the plasma concentration of this vitamin and of carotenes in middle-aged and old individuals are still scanty. The present investigation was undertaken with the purpose of supplying such information.

The study comprises analyses of plasma total carotenes, $\alpha + \beta$ carotenes and vitamin A in 155 individuals (71 men and 84 women) between the ages of 40 and 99. For comparison 47 younger individuals (22 men and 25 women) between 16 and 39 years were included in the investigation. The majority of the older subjects were inmates or patients in the St. Louis City Infirmary and City Infirmary Hospital, whereas the greater part of the younger individuals examined were private volunteers. The study was undertaken during the months July to September, 1947. During this period the diet offered to the patients in the 2 above-mentioned institutions

[1] Funds and materials provided by Hoffmann La Roche, Inc.

was calculated to contain an average of 2,000 and 4,000 μg of vitamin A (vitamin A + carotene) daily, a content which is considered adequate for adult individuals. None of the patients examined was given supplementary vitamin preparations during the weeks preceding the blood analysis. The vitamin content of the diet of the private group of younger individuals was not evaluated.

METHODS

The technique used was a modification of those recommended by The Association of Vitamin Chemists (Methods of Vitamin Assay, '47), Willstaedt and With ('38) and With ('47). The carotenes and vitamin A were extracted from the saponified plasma sample by successive portions of ether. After evaporation of the ether, the residue was dissolved in petroleum ether. The total carotenes were determined by use of a Dubosque colorimeter, and the $\alpha + \beta$ carotenes after previous adsorption on an aluminum oxide column and subsequent elution with benzol-benzene (fluid chromatography). The vitamin A was estimated on aliquots of the petroleum ether sample by the Carr-Price reaction. The more detailed technique will be given below. It will be noted that the customary treatment of the combined ether extracts with anhydrous sodium sulfate was omitted in the authors' procedure. This omission was undertaken because it was found that several samples of sodium sulfate examined yielded substances of a marked yellow color, which, furthermore, gave a strong Carr-Price reaction, thus interfering with both the carotene and the vitamin A determinations. Only after the exclusion of this step of the analysis were reproducible results obtained with constancy. It appears likely that this precaution accounts for the somewhat low vitamin A plasma values reported in this investigation. In order to avoid turbidity in the Carr-Price color development due to traces of water in the sample, a few drops of acetic anhydride (as recommended by With, '47) were added to the chloroform

solution before the addition of the antimony trichloride reagent. This practically always resulted in samples completely free from turbidity. As only samples that were absolutely clear were included in the study this precaution may also tend to lower the vitamin values reported in this paper.

Analytical technique

A blood sample of 50 ml was drawn in the morning from the fasting individual. Coagulation of the blood was prevented by potassium oxalate. After centrifugation 20 ml of plasma were measured into a 125 ml Erlenmeyer flask with a ground glass joint and 20 ml of 95% alcohol and 4 ml of 60% potassium hydoxide added. A water-cooled reflux condensor was inserted and the sample brought to boiling on an electric plate. After 15 minutes of boiling, the sample was cooled and transferred to a 125 ml separatory funnel containing 10 ml of water. The saponification flask was rinsed with 2 successive portions of 15 ml of water and 3 portions of 25 ml of ether, the washings being added to the sample in the separatory funnel. The funnel was agitated gently for 1 minute, after which the layers were allowed to separate. As a rule, less than 10 minutes were required for complete separation. After separation the lower, aqueous layer was drained off into another separatory funnel and re-extracted by shaking with 2 successive portions of 25 ml of ether, each ether sample after separation being added to the first ether extract. The combined ether extracts [2] were then washed in the separatory funnel with 2 successive portions of 15 ml of water. The separation of the ether layer from the water was usually accomplished in a few minutes; in those instances where the separation occurred more slowly the addition of a few milliliters of alcohol usually hastened the separation. After the last washing the ether was transferred to a 250 ml beaker

[2] It was found that treatment with 3 successive portions of ether sufficed for the complete extraction of the vitamin A from plasma samples to which enough vitamin A had been added to bring the vitamin concentration to above 300 μg %.

placed on an electric plate and the solvent evaporated by employment of gentle heating and constant application of a current of nitrogen from a cylinder. The residue was dissolved in petroleum ether and made up to 10 ml volume.

For determination of *total carotenes* the sample was compared in a Dubosque colorimeter with a standard carotene solution, containing 200 μg of β carotene in 100 ml of petroleum ether.

The α + β *carotenes* were determined by fluid chromatography and subsequent colorimetry as outlined above. For adsorption of the carotenes a column of aluminum oxide[3] was prepared, approximately 50 mm long and 2.5 mm wide, through which 2.5 ml of the petroleum ether sample was passed under gentle suction. The elution of the α + β carotenes was performed by addition of 3–4 ml of a mixture of equal volumes of benzole and petroleum ether, which was passed through the column in 1 ml portions. The first of these portions usually contained all the eluted carotene, the subsequent samples being colorless. For estimation of the α + β carotene value the eluant was made up to a proper volume and compared in the Dubosque colorimeter with the above-mentioned carotene standard solution. The standard deviation of the α + β carotene measurements was found to be 10 to 15 μg %.

The determination of *vitamin A* was performed in duplicate on two 3.5 ml portions of the petroleum ether solution. The samples were pipetted into calibrated test tubes fitted for use in a photocolorimeter. The petroleum ether was evaporated in a water bath under application of a strong current of nitrogen. The residue was dissolved in 1.7 ml redistilled chloroform, after which 5 drops of acetic anhydride were added. After placing the tubes in the colorimeter 8.3 ml of a 25% solution of antimony trichloride in chloroform were rapidly added and the light transmission measured within a few seconds after the addition. The vitamin A value was

[3] Alumina adsorption, Fisher Scientific Company.

obtained from a curve constructed by measurements on samples from a standard solution of vitamin A alcohol in chloroform.[4] The vitamin A values obtained from the curve were corrected for the color yielded by carotene in the Carr-Price reaction, using the following formula (Hawk, Oser and Summerson, '47):

$A - \frac{C}{20}$ = micrograms of vitamin A per 100 ml of plasma, where A is the concentration of apparent vitamin A and C that of carotene, both expressed per 100 ml of plasma.

As it appears debatable whether the correction for total carotenes or $\alpha + \beta$ carotenes gives the truest vitamin A value both the corrected figures will be given in the report of the analyses.

The standard deviation of the vitamin A estimation was found to be 2.6 μg per 100 ml of plasma.

EXPERIMENTAL

In figure 1 the vitamin A values are plotted graphically in relation to the age of the individuals. Table 1 contains the summarized carotene and vitamin A values for the age groups 16–39, 40–59, 60–69, 70–79, and 80–89 years, and the values for the men and women separately.

It will be seen from the reported data that both the carotene and the vitamin A values show great variation in the different individuals. It will further be noted that the average concentrations of plasma total carotenes and $\alpha + \beta$ carotenes are higher (320 and 190 μg %) in the younger individuals (16–39 years) than in the middle-aged and older groups. An analysis of the figures reveals, however, that the difference between the means is of doubtful significance, since it is smaller than the standard deviation of the difference.

[4] As solutions of the crystalline vitamin A alcohol in chloroform deteriorate quickly (in contrast to vitamin A in plasma extract preparations) the photoelectric determinations necessary for construction of the standard curve were performed immediately after the preparation of the solutions.

The average concentrations for vitamin A, total carotenes, and $\alpha + \beta$ carotenes in plasma were 17–23, 200–320, and 100–190 μg %, respectively. No certain difference was found between the male and female subjects studied.

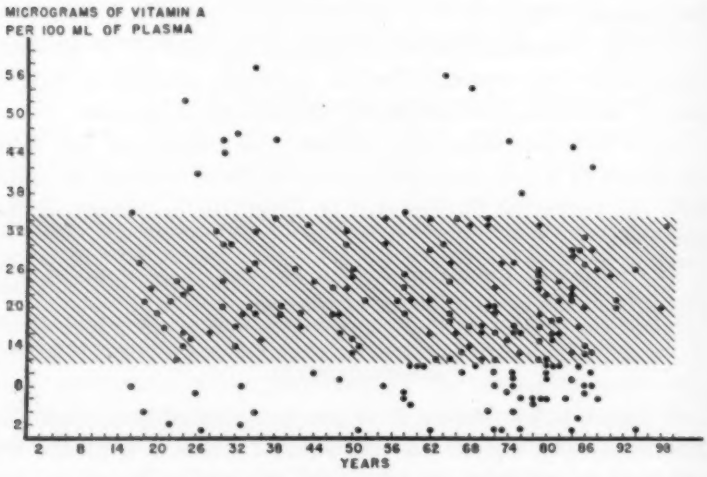

Fig. 1 The vitamin A values of plasma in 202 individuals. Correction for total carotenes was applied. The hatched area indicates values within one standard deviation from the mean observed in the younger age group.

SUMMARY

An investigation was made of the concentrations of plasma vitamin A, total carotene and $\alpha + \beta$ carotene in 155 middle-aged and old individuals offered a diet adequate in vitamin A, and in 47 younger subjects. The individual values observed varied greatly, but no statistically significant differences were found between the calculated means of the various age groups. The average concentrations of vitamin A, total carotenes, and $\alpha + \beta$ carotenes in plasma were 17–23, 200–320, and 100–190 μg %, respectively. No certain difference was found between the male and female subjects studied.

TABLE 1

Total carotene, α + β carotene, and vitamin A values of plasma in 202 individuals

AGE GROUP		NUMBER OF INDIVIDUALS	TOTAL CAROTENES		S.D.[1]	α + β CAROTENES		S.D.[1]	VITAMIN A [2]		S.D.[1]	VITAMIN A [3]		S.D.[1]
			Range of individual values	Mean value		Range of individual values	Mean value		Range of individual values	Mean value		Range of individual values	Mean value	
			Micrograms per cent			Micrograms per cent			Micrograms per cent			Micrograms per cent		
16–39	Total	47	120–540	320	91	70–380	190	77	1–57	23	13.4	3–64	30	14.7
	Male	22	120–540	320	113	70–380	190	94	1–57	23	13.0	3–64	30	14.2
	Female	25	170–460	320	70	70–300	180	61	2–47	22	13.6	7–58	30	15.0
40–59	Total	34	100–380	220	87	30–270	120	61	1–35	20	8.9	6–41	25	9.4
	Male	19	100–380	220	88	40–270	120	58	6–35	21	7.9	11–41	26	8.8
	Female	15	100–380	230	85	30–250	120	64	1–32	17	9.4	6–40	23	10.2
60–69	Total	25	70–320	200	67	20–210	110	53	1–56	22	12.8	3–61	27	13.6
	Male	15	70–310	190	74	40–210	120	56	1–56	22	15.7	3–61	27	16.3
	Female	10	110–320	200	61	20–160	90	41	12–33	21	6.8	14–41	28	8.6
70–79	Total	45	40–440	210	91	20–260	110	53	1–46	17	10.5	4–58	23	11.8
	Male	22	70–400	200	89	30–240	100	55	1–33	13	8.1	4–39	18	8.7
	Female	23	40–440	210	93	20–260	110	51	1–46	22	11.0	7–58	28	12.8
Above 80	Total	51	80–410	210	87	20–240	110	55	1–45	18	9.5	5–49	23	9.5
	Male	15	100–410	200	80	40–240	120	58	1–40	19	11.0	5–45	24	11.0
	Female	36	80–380	210	89	20–220	100	53	1–45	17	8.9	5–49	23	9.2

[1] Standard deviation.

[2] Correction applied for total carotenes.

[3] Correction applied for α + β carotenes.

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STORAGE BY BOBWHITE QUAIL OF VITAMIN A FED IN VARIOUS FORMS

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Two forms of true vitamin A are found in animals, the alcohol that normally circulates in the blood stream, and the ester that comprises the bulk of vitamin A stores in the liver (Glover, Goodwin and Morton, '47). Other esters, including vitamin A acetate, do not occur naturally, but have been synthesized in the laboratory.

In the plant kingdom true vitamin A is non-existent, but instead there are certain carotenoid pigments that are converted to vitamin A by animals. Theoretically, 3 of these pigments, namely, alpha and gamma-carotene, and cryptoxanthin, are changed to vitamin A molecule for molecule; whereas a fourth, beta-carotene, will produce 2 molecules of vitamin A for each molecule of the pro-vitamin. Actually, such an efficient conversion of the carotenoids does not exist.

The biological value of carotene is much lower than that of vitamin A (Ewing, '41). Ahmad and Malik ('33) and Rosenberg ('45) pointed out that animals differ radically in their abilities to synthesize vitamin A from carotene. Kemmerer and Fraps ('38) maintained that not only does the utilization of carotene depend on the kind of animal involved, but also on the quantity of carotene fed and the nature of the carrier. Carotene in cottonseed oil, for example, was digested twice as efficiently by rats and chickens as carotene

in alfalfa leaf-meal. According to Russell ('39) greater assimilation of carotene takes place on a high-fat diet than on a low-fat diet.

Studies by Nestler ('46) have shown that the bobwhite quail should be included in the list of species utilizing vitamin A more efficiently than carotene. Quail maintained on an unsupplemented winter-holding diet had a survival rate equal to that of birds maintained on the same diet supplemented with 500 I.U. of carotene per pound (⅛ of the birds' requirements for optimum maintenance and subsequent reproduction), while the survival rate for birds on the basal diet supplemented with 500 I.U. of vitamin A was more than twice as great. The hatchability of eggs, and the survival of offspring were significantly higher for quail maintained on vitamin A-supplemented diets than for birds receiving an equivalent number of units of carotene. Also, the liver storage of vitamin A in both growing stock and birds being maintained through the winter on diets containing vitamin A and carotene in excess of body-requirements, was 3 to 15 times greater from the former than from the latter.

During the winter of 1945-46, the livers of 59 wild quail killed in 4 eastern states showed an average storage of vitamin A of over 300 I.U. per gram. Hence, several questions have arisen. Did this stored surplus come from carotene alone, or from a mixture of carotene and vitamin A? Would massive intakes of carotene promote storage of vitamin A in pen-reared quail to the same extent as that found in the wild birds? Would the precursor in a natural carrier like alfalfa leaves be assimilated by quail as efficiently as crystalline carotene in cottonseed oil? Can the 3 forms of true vitamin A, the alcohol, the natural ester, and the "synthetic" acetate, be utilized by quail from the diet to the same degree? During 1946-47, 3 experiments involving 236 quail chicks, were conducted at the Patuxent Research Refuge, Laurel, Maryland, to answer these questions.

PROCEDURE

The basal feed mixture used in these experiments had the following percentage composition: ground white corn 33.38, soybean oil meal 51.00, dried skim milk 10.00, butyl fermentation flavin concentrate 2.00, steamed bonemeal 1.86, ground limestone 0.56, salt mixture [1] 1.00 and vitamin D-activated animal sterol 0.20.

The experimental diets consisted of this basal mixture supplemented by the addition of vitamin A. Fresh batches of each experimental diet were prepared each week during the experimental period, and the vitamin A activity of each diet and of each supplement was determined spectrophotometrically at frequent intervals to insure that the levels of vitamin A intake remained constant. In experiment 1, each diet contained 3000 I.U. of vitamin (or pro-vitamin) A per pound of feed, the amount shown by Nestler ('46) as meeting the quail's requirements for maximum growth. In experiment 2, the diets contained 25,000 I.U. per pound, or more than 8 times the quantity required for maximum growth.

Five forms of the vitamin were compared. Diet 1 contained vitamin A ester from natural sources; diet 2, vitamin A acetate; diet 3, vitamin A alcohol; diet 4, crystalline carotene (90% beta-10% alpha); diet 5, a mixture of vitamin A ester and crystalline carotene, each of which provided half the units. Each form was dissolved in sufficient cottonseed oil to establish a potency of 2000 I.U. per gram of solution. These diets were fed ad libitum to pens of 18 or 19 quail chicks during the first 10 weeks of life.

Three diets were compared in experiment 3, each one being fed ad libitum to 12 birds for the first 10 weeks of life. Each mash contained 5000 I.U. of vitamin (or pro-vitamin) A per pound of feed: Diet 1, vitamin A acetate; diet 2, crystalline carotene in cottonseed oil; and diet 3, carotene of dehydrated alfalfa leaf meal.

[1] The salt mixture consisted of 100.0 parts of iodized sodium chloride plus 1.7 parts manganous sulfate.

At the close of each experiment birds were sacrificed, 6 from each diet of experiments 1 and 2, and 5 from each diet of experiment 3. The livers were assayed spectrophotometrically for vitamin A by use of a modification of the technique of Wilkie and DeWitt ('45), those of the first 2 studies as individual samples, but those of the last study as composite samples, one per diet. The data from experiments 1 and 2 were analyzed statistically by Fisher's ('32) "t" and "2" tests.

RESULTS

The results are summarized in table 1.

TABLE 1

Storage of vitamin A in livers of growing quail at end of 10 weeks (I.U. per gram of liver)

EXP. NO.	LEVEL OF VIT. OR PROVIT. A (I.U./LB.)	AVE. AND RANGE OF STORAGE	FORM OF VITAMIN A					
			Vit. A ester	Vit. A acetate	Vit. A alcohol	Crystalline carotene	½ Carotene ½ Ester	Carotene of alfalfa leaf meal
1	3,000	Ave.	47	122	78	28	47	..
		Range	38–55	49–157	22–108	18–44	24–75	..
2	25,000	Ave.	2460	3966	1655	228	649	..
		Range	1084–3536	3038–5430	1471–2096	89–532	323–926	..
3	5,000	Ave.			244	..	24	52

Experiment 1

The differences between the storage from vitamin A acetate and that from any of the other supplements are highly significant. The difference between vitamin A alcohol and either the natural vitamin A ester or the combination of ester and carotene is according to odds of 19:1, whereas that between vitamin A alcohol and carotene is according to odds 99:1.

When the efficiency of utilization of crystalline carotene for storage by quail at the 3000 I.U. level in the diet is given the rating of 1, the other supplements receive the following

ratings: Combination of carotene and vitamin A ester, 1.7; vitamin A ester, 1.7; vitamin A alcohol, 2.8; and vitamin A acetate, 4.4.

Experiment 2

Again the difference between the storage from vitamin A acetate and that from any of the other supplements is highly significant. This time, however, the results on natural vitamin A ester are greater than those on vitamin A alcohol (odds of 19:1), or those from either carotene, or the combination of carotene and the ester (odds of 99:1). The difference between the storage from vitamin A alcohol and that from either carotene or the combination, is highly significant.

If, at the 25,000 I.U. level in the diet, carotene is rated as 1 for storage, then the other ratings would be as follows: Combination of carotene and vitamin A ester, 2.8; vitamin A alcohol, 7.3; vitamin A ester, 10.8; and vitamin A acetate, 17.4.

At the high level of vitamin A considered in this study, as much as 530 I.U. of the factor were stored per gram of liver from carotene by some quail during the first 10 weeks of life.

Experiment 3

The storage of vitamin A from the carotene of alfalfa leaf meal was nearly double that from the crystalline carotene in cottonseed oil. The storage from the vitamin A acetate, on the other hand, was over 4½ times as great as that from the carotene of the alfalfa, and over 10 times as great as that from crystalline carotene.

DISCUSSION

Quail, after the first few weeks of life, are primarily granivorous and to some extent frugivorous. Handley's ('31) analysis of 1659 quail-crops disclosed the fact that only 14.5% of the food consisted of animal matter, mostly insects. According to Massey ('38) less than 5% of animal food is consumed by quail during winter months.

Analysis at Patuxent Research Refuge of 40 species of small animals that might be used as food by quail, revealed no evidence of vitamin A except in 4 cases, namely, earthworms, spiders, spittle bug larvae, and grain weevil larvae. However, in the light of additional tests, there is considerable doubt about even those 4 species possessing any true vitamin A. On the other hand, herbivorous small animals, such as grasshoppers, Colorado potato beetles, Mexican bean beetles, tent caterpillars, and the like, were found to have high concentrations of carotene, in some cases running as high as 230 I.U. per gram of entire carcass. Therefore, apparently the main, and possibly the sole, source of vitamin A for wild quail is carotene, obtained either directly from plants or indirectly from herbivorous insects and similar small animals.

SUMMARY

According to studies conducted with 236 bobwhite quail chicks at Patuxent Research Refuge, crystalline carotene in cottonseed oil fed at levels of 3000 I.U. (the requirement for optimum growth), 5000 I.U., and 25,000 I.U. per pound of feed, was utilized only ⅓ to ⅐ as efficiently as vitamin A alcohol; ½ to 1/10 as natural vitamin A ester; and ¼ to 1/17 as vitamin A acetate, based on the storage of vitamin A in the liver.

The carotene in the natural carrier, alfalfa leaf meal, was assimilated as effectively as was crystalline carotene in cottonseed oil, when both were fed at 5000 I.U. per pound of feed.

Crystalline carotene when fed at a level over 8 times the requirement of the quail for maximum growth, or 25,000 I.U. per pound of feed, was stored as vitamin A in the livers of pen-reared quail to an extent comparable to the levels found in certain young wild quail.

Vitamin A acetate was utilized more efficiently by quail than either vitamin A alcohol or the natural vitamin A ester.

There was considerable individual variation in storage of vitamin A by quail on the same diet, and with the same or similar parental background.

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